Syed Shadab Raza *Editor*

Regenerative Therapies in Ischemic Stroke Recovery

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"WE ARE BORN TO LOVE
LOVE IS OUR MOTHER"
RUMI

This book is dedicated to my mother, who has always been a source of inspiration for my work and success.
Syed Shadab Raza, PhD

Foreword by Prof. P. K. Seth

Stroke is a major cause of mortality and morbidity in developed countries, and the incidence of stroke is increasing in lower middle-income countries including India. Recently, some post Covid-19 deaths due to stroke have been reported globally. As per a recent systematic review, stroke is the fourth leading cause of deaths and fifth leading cause of disability in India. In this context, the book entitled *Regenerative Therapies in Ischemic Stroke Recovery*, so elegantly compiled and meticulously edited by Dr. Syed Shadab Raza, assumes a great significance. The book aims to provide a broad overview of the current state of integrative regenerative techniques and the recent advances in this field leading into a state-of-the-art framework for understanding the potential of regenerative therapy for ischemic brain. The key aspects of the use of stem cells and nanomedicines in understanding the processes underlying the benefits of regenerative therapies in stroke pathology have been beautifully covered in this book.

The editors and contributing authors are well-known experienced colleagues from India and other nations, who have made significant original contributions, demonstrated by their ability to identify needs, describe technologies, and showcase their application(s). They point to clinical research's existing and future importance in the regeneration field by summarizing and analyzing significant results from a fundamental study into the translational application. The coverage includes the key areas of regenerative medicines: the use of miRNA, exosomes, and nanoparticles in addition to stem cells throughout diagnosis, repair, and regeneration, with a particular focus on techniques and interventions aimed at ischemic brain regeneration.

Applying these cutting-edge technologies and methodologies will aid readers in generating innovative strategies/targets for developing simple-to-use, cost-effective diagnostic techniques and successful therapeutics.

I commend both the authors and the editors on their excellent work and feel confident that this will serve as a research-based reference book for biomedical scientists, researchers, teachers, and others in the healthcare industry who work on various facets of neurodegenerative diseases, and they will find it extremely useful. Also, the students from many disciplines interested in different elements of current and emerging neurotransplantations and regenerative medicine will enjoy reading this book. I feel privileged to have the opportunity to write the foreword for this important book which provided me a good deal of new information on the subject.

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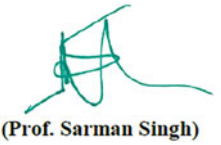
Stroke is the major cause of morbidity and mortality worldwide, with developing countries accounting for 40% of all strokes. Regenerative medicine, which includes stem cells, nanomedicines, and other therapies, claims to be promising treatment option for stroke patients. A new era has begun! Here is a book titled ***Regenerative Therapies in Ischemic Stroke Recovery***, compiled by a mid-career scientist ***Dr. Syed Shadab Raza***, which focuses on regenerative therapy, options available for stroke rehabilitation, and its future directions. The book is written by active research experts in the subject area. The chapters contain valuable knowledge and advances at the molecular level in numerous areas related to advancing regenerative medicine in stroke rehabilitation. The beauty of this book is that it brings stem cells and nanotechnology together under one head, providing a comprehensive picture of

breakthroughs in stem cells and nanotechnology and their practical usage in treating brain stroke over the last two decades.

This book elucidates the significance of miRNAs, exosomes, and other regenerative medicine tools in treating stroke. Stem Cells, Adult Stem Cells, Embryonic Stem Cells, induced Pluripotent Stem Cells, Nanomedicine, Polycaprolactone, and Poly Lactic Glycolic Acid nanoparticles, etc. This book contains authoritative evaluations of a range of stem cell systems and nanomedicine by international experts. The book, in my opinion, will benefit biologists interested in human and veterinary medicine.



Bhopal, India
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Sarman Singh

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Syed Shadab Raza is an associate professor and head of the Department of Stem Cell and Regenerative Medicine, Era's Lucknow Medical College and Hospital, India. He is also serving as an assistant dean of the Faculty of Basic Medical Science, Era University, and is a visiting scientist at the American University of Barbados, Latin America. His research focuses on translational (stem cell and nanotechnology) therapy for stroke recovery. Specifically, he is interested in exploring the potential of stem cell and nanoparticle therapy to treat ischemic stroke. He has received various prestigious awards, notably the International Brain Research Organization Fellowship, Japan Neuroscience Society Award, Indian Academy of Biomedical Sciences Neuroscience Award, InStem Fellowship, IBRO-ABRC Fellowship, and Council for Scientific Industrial and Research Fellowship. In the year 2020, Dr. Raza was elected as a Fellow of the Royal Society of Biology.

He is a member of the editorial board and reviewer for several prestigious journals of international repute, such as *Frontiers in Neuroscience*, *Frontiers in Pharmacology*, *Stem Cell Research and Therapy*, *World Journal of Stem Cells*, and *Recent Patents on Drug Delivery and Formulation*. He has published more than 41 research articles in peer-reviewed international journals and co-authored several book chapters. He is a member of several national and international scientific societies, such as the National Academy of Sciences India, Indian Society of Regenerative Science, Indian Stem Cell Study Group Association, Japan Neuroscience Society, Indian Science Congress Association, Indian Society of Cell Biology, Society for Neurochemistry India, and Indian Academy of Neuroscience.

Targeting Adult Neurogenesis for Brain Recovery After Stroke: The Next Frontier in Stroke Medicine

1

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Abstract

One in four people over age 25 will have a stroke in their lifetime. Globally, an estimated 80 million people are currently living with stroke with many experiencing chronic disability and unmet needs. There is strong evidence demonstrating that the brain has a remarkable capacity for plasticity and reorganization into adulthood; however, application of this knowledge clinically is in its infancy. Adult neurogenesis is the generation of de novo neurons from neural

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stem cells and the integration of these immature neurons into established circuits in the adult brain. Therefore, neurogenesis is a really promising therapeutic for stroke patients, and we are going to highlight the ways it can be exploited to improve stroke outcome in this chapter. Briefly, we outline what is known about adult neurogenesis, and the techniques typically used to investigate it in humans and preclinical studies. We then provide evidence of post-stroke neurogenesis from both clinical and preclinical studies. Finally, we discuss some potential pharmacological and non-pharmacological approaches to enhance post-stroke neurogenesis to promote stroke recovery.

Keywords

Brain plasticity · Brain repair · Neurogenesis · Neuroplasticity · Stroke recovery

1.1 Introduction

Stroke is a leading cause of death and disability worldwide. Globally, one in four people over age 25 will have a stroke in their lifetime, and there are over 13.7 million new strokes each year (Lindsay et al. 2019). Alarmingly, the incidence of stroke in people of working age (under 60 years old) has risen. Despite development of recanalization therapies, the number of stroke survivors is increasing with over 80 million people currently living with stroke globally. Almost all live with post-stroke complications, such as motor deficits and cognitive impairment, which may impede their ability to carry out activities of daily living. Therefore, it is necessary to develop new therapeutic strategies to improve functional outcomes after stroke.

Over the last few decades, major attention has been invested in neuroprotective therapies, which are designed to prevent or reduce cell death from ischemia. Initial preclinical studies demonstrated several drugs are effective for treating stroke in animal models; however, subsequent clinical trials have been disappointing, and none of the agents has proven effective in patients (O'Collins et al. 2005, 2017). Recently, neurorestoration has become a topic of interest since it was reported that the brain has the capability to repair itself after injury. The development of new therapeutic strategies to improve neurological function by enhancing the endogenous capacity for neurorestorative processes in the stroke damaged brain may help stroke patients with chronic disabilities.

For centuries, scientists believed that the central nervous system (CNS) was a fixed system and "hard-wired" once we reach adulthood. This dogma suggests that the brain is not capable of generating new cells after the development phase or regenerating after injury. Fortunately, this dogma has collapsed in recent years, with the discovery of adult neurogenesis in animal studies and human post-mortem brain samples. Excitingly, increased neurogenesis has been reported in adult animal models of stroke and even in stroke patients. However, with the number of "new-born" neurons being insufficient for full functional recovery and their existence being transitory, stroke-induced neurogenesis is thought to operate only for a limited

time period after stroke. In this chapter, we provide an overview of adult neurogenesis (Sect. 1.2), and the techniques used to investigate neurogenesis (Sect. 1.3). Next, we will present evidence of neurogenesis after stroke from both preclinical and clinical studies as well as discuss the mechanisms of stroke-induced neurogenesis (Sect. 1.4) and shortcomings of this repair process (Sect. 1.5). Finally, we will outline some promising strategies, both pharmacological and non-pharmacological, to enhance stroke-induced neurogenesis and promote stroke recovery (Sect. 1.6). The overall focus of this chapter is to critically appraise whether targeting adult neurogenesis following stroke is a potential therapeutic strategy.

1.2 Adult Neurogenesis

Adult neurogenesis describes the formation of de novo neurons from neural stem cells and the integration of these neurons into established circuits in the adult brain. This is a conserved process across multiple mammalian species. Neurogenesis occurs in two locations within the adult mammalian brain: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus, with slight differences in maturation processes in each region (Fig. 1.1). There are several phases of neurogenesis: proliferation, differentiation, migration, survival, and integration. It has been estimated that maturation takes approximately 7 weeks in total (Kempermann et al. 2015).

In the SVZ, quiescent neural stem cells (type B) share many features with astrocytes and are activated and differentiate into amplifying neuronal progenitors. These cells rapidly proliferate and differentiate into neuroblasts (Cutler and Kokovay 2020). Neuroblasts can be identified by the expression of doublecortin (DCX) and polysialyated neural cell adhesion molecule (PSA-NCAM). Neuroblasts migrate through chains of glial fibrillary acidic protein (GFAP)-positive cells in the rostral migratory stream from the lateral ventricle to the olfactory bulb. From the olfactory bulb, they move out of the rostral migratory stream and differentiate into interneurons. Subsequently, the majority of these differentiate into gamma-aminobutyric acid (GABA)- and calretinin-positive granule neurons or GABA-, calretinin-, calbindin-, and tyrosine hydroxylase-positive periglomerular cells. These neurons also express a marker of mature neurons, neuronal nuclei (NeuN). Type B cells can also give rise to oligodendrocytes and astrocytes (Abrous et al. 2005; Bath et al. 2008; Lim and Alvarez-Buylla 2016; Rikani et al. 2013).

In the SGZ, neurogenesis gives rise to granule neurons only. GFAP-, nestin-, and Sox2-positive radial glial-like neural stem cells differentiate into amplifying neural progenitor cells with high proliferative activity. These cells continue to express nestin and begin to express the first indications of neuronal differentiation including transcription factors NeuroD and Prox1 and DCX. These cells also begin to receive GABAergic inputs which at this stage are excitatory. As proliferation and differentiation into neuroblasts continues, nestin is downregulated and PSA-NCAM is upregulated. These cells exit the cell cycle and their lineage is determined as granule neurons. Immature granule neurons begin to express NeuN and calretinin while

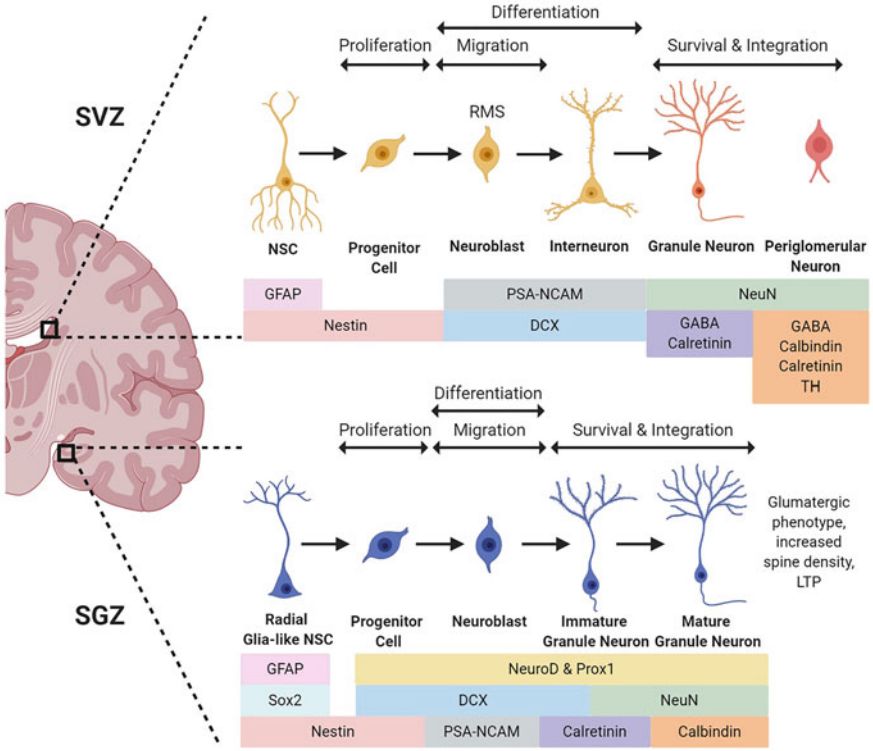

Fig. 1.1 Adult neurogenesis in the subventricular zone (SVZ; top panel) and subgranular zone (SGZ; bottom panel). The specific cell markers indicate the specific cell types generated at a particular phase during the process of neurogenesis. (Created with BioRender.com). Abbreviations: *NSC* neural stem cell, *GFAP* glial fibrillary acidic protein, *DCX* doublecortin, *PSA-NCAM* polysialyated neural cell adhesion molecule, *RMS* rostral migratory stream, *GABA* gamma-aminobutyric acid, *TH* tyrosine hydroxylase, *NeuN* neuronal nuclei, *LTP* long-term potentiation

undergoing morphological changes such as dendrite and axon extension into the CA3 and hilar regions of the hippocampus. There are initially a high number of NeuN-positive cells but many are quickly eliminated by apoptotic mechanisms. Cells that survive for 2 weeks appear to be stably and persistently integrated into circuits. As part of maturation, immature granule neurons develop a glutamatergic phenotype upon which GABA becomes inhibitory and glutamate becomes excitatory as in mature neurons. Maturing granule neurons develop mature dendritic spines and switch their calcium binding protein from calretinin to calbindin. Granule neurons also undergo a period of increased synaptic plasticity including long-term potentiation and within a few weeks are electrophysiologically indistinguishable from their older neighboring neurons (Goncalves et al. 2016). A summary of phases and markers of adult neurogenesis is presented in Fig. 1.1.

While adult neurogenesis has been well characterized in several mammalian species including rodents and nonhuman primates, there has been controversy over

its existence in humans in recent years. A report by Sorrels et al. (2018) concluded that neurogenesis in the human hippocampal dentate gyrus drops to undetectable amounts during childhood, and that the human hippocampus must function differently from that in other species, in which adult neurogenesis is conserved. However, Boldrini et al. (2018) came to the opposite conclusion and reported lifelong neurogenesis in humans. Thus, in the space of only a few weeks, two studies were published that could not be more different. Taking methodological approaches into consideration, our current view is in line with Boldrini et al. and other bodies of evidence that support the significance of adult neurogenesis in health and disease. Further, we suggest that endogenous post-stroke neurogenesis could be promoted with pharmacological and non-pharmacological intervention to improve stroke outcome.

1.3 Techniques to Investigate Adult Neurogenesis

There are several methods for studying adult neurogenesis in both rodents and humans. In this section, we will briefly introduce the principles of these techniques and discuss some caveats on how the results could be interpreted and their limitations.

1.3.1 Post-mortem/Histological Techniques

DNA Labeling The first evidence of adult brain neurogenesis came from Altman (1962), who labeled rodent nuclear DNA using [^{3}H]-thymidine, an exogenous marker of DNA replication. Once administered, [^{3}H]-thymidine is incorporated into DNA during the S-phase of the cell cycle and is detected using autoradiography. Despite supporting findings from Kaplan and Hinds (1977) and Bayer (1983), methodological doubts over this technique meant that their findings were not widely accepted. It was not until experiments using bromodeoxyuridine (BrdU; a thymidine analogue) by Nottebohm (1985) in canaries that people started to take the idea of adult neurogenesis more seriously. Although analogous to [^{3}H]-thymidine, the advantage of BrdU is that it is detectible using immunohistochemistry (IHC) which is considered to be a more robust method of detection than autoradiography. Furthermore, IHC permits a more in-depth analysis of tissue through the use of single, or double, labeling techniques making thymidine analogues, as opposed to [^{3}H]-thymidine, the preferred markers for assessing in vivo adult neurogenesis (Kuhn et al. 2016).

Protein Markers Proteins expressed by cells actively engaged in the cell cycle have been used as markers to identify differentiating neuronal cells at the time of tissue collection. For example, cyclin-dependent kinase-1 (Cdk1) and proliferating cell nuclear antigen (PCNA) are important regulators of the cell cycle and are expressed by cells engaged in it. Specific protein markers can also pinpoint the

phase of the cell cycle a cell is engaged in. Furthermore, targeting proteins specific to distinct phases of the cell cycle can provide temporal resolution on which cells are in which phase of the cell cycle, e.g., Ki-67 is expressed during all phases except quiescence and phosphohistone H3 (pHisH3) is expressed during G_2 phase. An advantage of using protein markers over thymidine analogues is that they do not require pre-emptive administration of the label prior to tissue collection. For a comprehensive review of endogenous proteins in adult neurogenesis, see Eisch and Mandyam (2007).

Protein markers of neuronal differentiation have also been used to investigate adult neurogenesis. DCX is a marker of immature neurons and is often favored as a protein marker due to its transient expression; neuroblasts begin to express DCX while they are dividing, and the daughter cells continue to produce it for 2–3 weeks. Downregulation of DCX occurs as upregulation of NeuN occurs when neuroblasts differentiate into mature neurons (Brown et al. 2003). However, it is often recommended to use DCX in combination with other markers as there is some evidence of its expression in neocortical neurons and NG2-positive cells (presumed to be glial progenitor cells). Some other markers that can be used include neuron-specific class III beta-tubulin (Tuj1), PSA-NCAM, and calretinin. It should be noted that there may be differences in the temporal profile of protein expression in different mammalian species. See Fig. 1.1 for a summary of protein expression in the SVZ and SGZ.

Viral Vectors An alternative labeling approach has been through the use of viral vectors to label proliferating cells with fluorescent reporter genes, e.g., green fluorescent protein (GFP). Retroviral vectors specifically infect dividing cells and introduce parts of their DNA into host DNA, thus inserting GFP into the genome of dividing neural progenitors. This can provide more long-term information than DNA labeling methods which are better for short-term quantification. For more information, see the review by Enikolopov et al. (2015).

Retrospective ^{14}C Dating Recent advances in mass spectrometry have permitted the analysis of ^{14}C in genomic DNA (Spalding et al. 2005). During mitosis ^{14}C is integrated into DNA with a concentration corresponding to atmospheric levels, effectively date stamping the DNA. When using this technique to investigate neurogenesis, neuronal cells are labeled (most commonly by NeuN) and separated from other cell types by flow cytometry. Neuronal DNA is then extracted and sent for spectral analysis. As opposed to the above techniques, radiocarbon dating is best for longer-term studies with the sensitivity of the technology between months to years. However, it only provides the average of neurons in the analyzed tissue; therefore, it does not permit investigation of individual neurons.

1.3.2 In Vivo Techniques

Magnetic Resonance Imaging (MRI) Recent advances in imaging technologies have allowed for examination of adult neurogenesis in vivo using MRI. Advantages of live imaging includes minimal to no side effects and the ability to perform longitudinal imaging studies. MRI relies heavily on correlations and the specificity for identifying neurogenesis has yet to be adequately validated in human and animal studies. An example of this is using cerebral blood volume as a marker of neurogenesis (Pereira et al. 2007). Neurogenesis can be indirectly assessed using cerebral blood volume measurements, due to the correlation between neurogenesis and angiogenesis. However, this coupling has yet to be confirmed in anything other than exercise.

1.4 Evidence of Adult Neurogenesis After Stroke: Preclinical and Clinical

Liu et al. (1998) were the first to report increased hippocampal neurogenesis after transient global ischemia in gerbils in 1998. The study showed that neuroblasts with neuronal features were first seen 26 days after ischemia, and survived for at least 7 months. These initial results led to multiple studies that confirmed increased neurogenesis in the SVZ and SGZ following ischemia in different preclinical stroke models, and in various species such as mice (Table 1.1), rats (Table 1.2), and nonhuman primates (Koketsu et al. 2006; Tonchev et al. 2003a, b, 2005).

In preclinical studies, significant increased proliferation of neural stem cells in the SVZ has been observed within the first 2 weeks following cerebral ischemia and persisted up to 4 weeks post-ischemia in both mice (Kreuzberg et al. 2010) and rats (Arvidsson et al. 2002; Jin et al. 2001; Parent et al. 2002; Zhang et al. 2004a). Furthermore, there is extensive evidence of SVZ neuroblasts migrating to sites of injury where they participate in brain repair and functional recovery (Hou et al. 2008; Jin et al. 2003b; Kreuzberg et al. 2010; Parent et al. 2002; Yamashita et al. 2006). Thored et al. (2006) demonstrated that the SDF-1α/CXCR4 signaling pathway drives migration of DCX+ neuroblasts toward ischemic damage where they aid in motor recovery. Further, conditional depletion of DCX+ neuroblasts prior to experimental stroke impaired long-term motor outcomes (Wang et al. 2012). Although repopulation of neurons within the hippocampus after stroke has been demonstrated in studies of both mice (Seong et al. 2018; Tureyen et al. 2004) and rats (Jin et al. 2001), the involvement of SGZ neurogenesis in post-stroke cognitive outcomes is a debatable topic. While post-stroke SGZ neurogenesis could promote cognition function (Sun et al. 2013), several studies have observed abnormalities in the morphology of new neurons concomitant to deficits in hippocampus-dependent tasks (Cuartero et al. 2019; Niv et al. 2012). This issue will be further discussed in the next section.

In humans, neurogenesis has been shown to begin within days after stroke regardless of type and location (Table 1.3). Macas et al. (2006) showed that stroke

Table 1.1 Studies investigating post-stroke adult neurogenesis in mouse models

References	Strain	Stroke model	Aims/objectives	Time post-stroke	Neurogenesis markers	Results/findings
Cuartero et al. (2019)	C57BL/6/ nestin-CreERT2/ NSE-DTA	MCAO	Determine if post-stroke neurogenesis contributes to the development of long-term memory impairment	7, 8, 35, and 65 days	IHC: BrdU NeuN DCX	Neurogenesis in the hippocampus continues 1 month after stroke and correlates with an impaired contextual and spatial memory performance
Kreuzberg et al. (2010)	5HT3A-EGFP	MCAO	Determine the contribution of SVZ neural progenitor cells to cortical neurogenesis following focal cerebral ischemia	14 and 35 days	IHC: BrdU EGFP DCX PSA-NCAM Nestin Musashi	Stroke increases the neuroblast production in the SVZ in the ipsilateral and contralateral hemisphere. Neuroblasts migrate to the cortex and can be detected 35 days after the infarct. Cortical SVZ-derived neuroblasts, generated in response to the stroke, differentiate into mature neurons
Seong et al. (2018)	C57BL/ 6TLR2 KO	Photothrombotic stroke	Examine the role of TLR2 in adult neurogenesis from NSC of hippocampal DG in cerebral ischemia	1, 3, 10, and 28 days	IHC: BrdU DCX NeuN	TLR2 promote adult neurogenesis from neural stem cell of hippocampal DG through increasing proliferation, differentiation, and survival from neural stem cells after ischemic injury of the brain
Tureyen et al. (2004)	C57BL/6	MCAO	Determine the proliferation, survival, and maturation of neural progenitor cells in the DG following transient focal cerebral ischemia	7 and 21 days	IHC: BrdU DCX NeuN	Focal ischemia induces neurogenesis in the DG of the mouse brain

Wang et al. (2012)	DCX-TK	MCAO	Examined the effect of NSC depletion on long-term anatomic and functional outcome from MCAO	1, 3 days, 1, 2, 4, 8, and 12 weeks	IHC: BrdU DCX	Acute post-ischemic neurogenesis exerts a persistent beneficial effect on outcome
Woitke et al. (2017)	C57BL/6	MCAO	Determine if new neurons are generated in the DG with focal infarcts and if they reveal specific hippocampus-dependent memory deficits	7 weeks	IHC: GFP RFP NeuN	Neurogenesis is significantly increased after MCAO. Following MCAO, mice show a significant impairment relating to the hippocampus-dependent memory tasks
Yamashita et al. (2006)	Wild-type ICR	MCAO	Determine the source of neuroblasts following focal cerebral ischemia	14, 18, 21, and 35 days	IHC: DCX NeuN GFAP	Neuroblasts originate in the SVZ and migrate toward the brain region infarcted by cerebral ischemia, where they differentiate into mature neurons

Abbreviations: *MCAO* middle cerebral artery occlusion, *IHC* immunohistochemistry, *BrdU* Bromodeoxyuridine, *NeuN* neuronal nuclei, *DCX* doublecortin, *SVZ* subventricular zone, *TLR2* toll-like receptor 2, *DG* dentate gyrus, *GFAP* glial fibrillary acidic protein, *EGFP* enhanced green fluorescent protein, *PSA-NCAM* highly polysialylated neural cell adhesion molecule

Table 1.2 Studies investigating post-stroke adult neurogenesis in rat models

References	Strain	Stroke model	Aims/objectives	Time post-stroke	Neurogenesis markers	Results/findings
Arvidsson et al. (2002)	Wistar	MCAO	Determine if new neurons are formed in the striatum following transient MCAO	4 weeks	IHC: BrdU DCX NeuN GFAP Vimentin	Transient MCAO in adult rats leads to a marked increase of cell proliferation in the SVZ. Stroke-generated new neurons and neuroblasts migrate into the severely damaged area of the striatum, where they express markers of developing and mature, striatal medium-sized spiny neurons
Darsalia et al. (2005)	Wistar	MCAO	Determine if MCAO triggers increased neurogenesis in the damaged striatum and non-damaged hippocampus of young and aged rats	7 weeks	IHC: BrdU NeuN DCX	Basal neurogenesis is impaired in the SGZ and the SVZ of aged animals, but both regions react to stroke with increased formation of new neurons. The magnitude of striatal neurogenesis after stroke is similar in both young and old animals, thus implying that this potential mechanism for self-repair also operates in the aged brain
Gu et al. (2000)	Wistar	Reversible photothrombotic stroke	Investigate the cell proliferation process in the cortical region-at-risk after focal cerebral ischemia	2, 3, 7, and 100 days	IHC: BrdU NeuN GFAP	New neurons can be generated in the cerebral cortex after sublethal focal cerebral ischemia

Hou et al. (2008)	Sprague-Dawley	MCAO	Investigate if GABAergic and cholinergic new neurons could differentiate into functional cells following focal cerebral ischemia	3 days, 1, 2, 4, 8, and 13 weeks	IHC: BrdU EGFP NeuN Map-2	Newly formed striatal GABAergic and cholinergic neurons following ischemia can become functionally integrated into neural networks in the brain of adult rats
Jiang et al. (2001)	Wistar	MCAO	Determine the occurrence of cortical neurogenesis after focal cerebral ischemia	30 and 60 days	IHC: BrdU Map-2 NeuN βIII-tubulin	The results showed that new neurons can be generated in the frontal, temporal, and parietal cortex, as well as the striatum after transient focal cerebral ischemia
Jin et al. (2001)	Sprague-Dawley	MCAO	Determine the effect of focal cerebral ischemia on neurogenesis in SGZ and SVZ	1, 2, and 3 weeks	IHC: BrdU NeuN PCNA DCX	Cerebral ischemia promotes neurogenesis in both SGZ and SVZ. Both effects were bilateral. Nevertheless, the effects in SGZ were more prominent on the ischemic side
Jin et al. (2003b)	Sprague-Dawley	MCAO	Investigate the migratory fate of cells arising through ischemia-induced neurogenesis in the brain following focal cerebral ischemia	8, 24, and 72 h, 2 weeks	IHC: BrdU DCX Nestin NeuN NeuroD βIII-tubulin ENCAM TUC-4	The results show directed migration of subventricular neuronal precursors into both the striatum and cortex of ischemic brain lesions
Jin et al. (2004)	Fisher-344	MCAO	Determine the effect of focal cerebral ischemia on neurogenesis and migration of newborn neurons in the young and aged rodents	24 h	IHC: BrdU DCX Nestin	The aged rat brain is more sensitive than the young adult brain to ischemic neuronal damage. Nevertheless, ischemia-induced neurogenesis persists in the SVZ of aged rats

(continued)

Table 1.2 (continued)

References	Strain	Stroke model	Aims/objectives	Time post-stroke	Neurogenesis markers	Results/findings
Niv et al. (2012)	Wistar	MCAO, Photothrombotic stroke	Determine the morphological properties of granule cells that are born and develop after the ischemic insult. Determine if these adult-born neurons properly integrate into the preexisting hippocampal circuitries	6 weeks	IF: CAG-GFP NeuN	Focal brain ischemia impairs correct morphological integration of newly generated neurons in the DG. A certain fraction of newborn neurons displayed aberrant features involving bipolar dendritic arborizations and ectopic location
Parent et al. (2002)	Sprague-Dawley	MCAO	Determine if focal ischemic injury would increase SVZ neurogenesis and direct migration and neuronal differentiation of endogenous precursors in damaged regions	10, 21, and 35 days	IHC: BrdU DCX	Transient MCAO increases SVZ cell proliferation and neurogenesis ipsilateral to infarcted brain regions, with many SVZ neuroblasts extending to sites of injury. Moreover, in damaged neostriatum, neuroblasts differentiate into cells that have neuronal morphologies and express markers specific for medium spiny neostriatal neurons
Sato et al. (2001)	Wistar	MCAO	Investigate the effect of aging on the migration of neural stem cell after brain ischemia	4 h, 1, 3, and 7 days	IHC: PSA-NCAM	Following transient MCAO, there is neurogenesis in both young and old rats. Nevertheless, the temporal expression was different between young and older rats

Takasawa et al. (2002)	Wistar	MCAO	Determine the proliferation of neural progenitor cells and cell survival after proliferation and neuronal differentiation of the nonischemic area after ischemia	1 and 28 days	IHC: BrdU	Focal ischemia stimulates the proliferation of neural progenitor cells but does not support their survival in the contralateral hippocampus
Thored et al. (2006)	Wistar	MCAO	Determine occurrence of striatal neuroblasts following stroke and explore if SDF-1α/CXCR4 signaling is involved in the migration of striatal neuroblasts following ischemia	1, 2, 6, 8, 12, and 16 weeks	IHC: BrdU DCX NeuN GFAP	Striatal neuroblasts are generated for 4 months following stroke. Neuroblasts formed early or late following stroke either differentiate into mature neurons, which are capable of surviving for several months, or die through caspase-mediated apoptosis. The SDF-1α/CXCR4 signaling pathway regulates the directed migration of new striatal neurons toward the ischemic damage
Zhang et al. (2001)	Wistar	MCAO	Examine the proliferation and fate of neural progenitor cells in the SVZ and DG after focal cerebral ischemia	Part 1–7, 14 and 21 days Part 2–1 and 28 days	IHC: BrdU PSA-NCAM NeuN GFAP MAP2	Following focal ischemia, there is increase in numbers of proliferating cells in the ipsilateral cortex, the SVZ and OB, but not in the DG, implying that cell proliferation is region specific. Proliferating cells may migrate from the SVZ to the OB. Focal ischemia does not induce neurogenesis in the cortex and subcortex

(continued)

Table 1.2 (continued)

References	Strain	Stroke model	Aims/objectives	Time post-stroke	Neurogenesis markers	Results/findings
Zhang et al. (2004a)	Wistar	MCAO	Investigate if neural progenitor cells in the adult SVZ of rats contribute to stroke-induced increase in neurogenesis	7, 14, and 21 days	IHC: BrdU DCX βIII-tubulin GFAP	Increase in recruitment of neural stem cells in the SVZ correlates with the increased neurogenesis observed following stroke. Furthermore, newly generated neurons migrate from the SVZ to the ischemic striatum

Abbreviations: *MCAO* transient middle cerebral artery occlusion, *IHC* immunohistochemistry, *h* hours, *BrdU* bromodeoxyuridine, *DCX* doublecortin, *NeuN* neuronal nuclei, *GFAP* glial fibrillary acidic protein, *SVZ* subventricular zone;,*CA1* hippocampal cornu ammonis, *SGZ* subgranular zone, *EGFP* enhanced green fluorescent protein, *PCNA* proliferating cell nuclear antigen, *ENCAM* embryonic nerve cell adhesion molecule, *DG* dentate gyrus, *NSC* neural stem cells, *NPC* neural progenitor cells, *PSA-NCAM* highly polysialylated neural cell adhesion molecule, *SDF-1α* stromal cell-derived factor-1α, *OB* olfactory bulb, *GABA* gamma-aminobutyric acid

Table 1.3 Neurogenesis following stroke in humans

References	Stroke patients	Stroke type/ location	Aims/objectives	Time post-stroke	Neurogenesis markers/ Technique	Results/findings
Jin et al. (2006)	4 males, 2 females; aged 34–74	Cerebral cortical infarcts	Determine if the human brain can repair through neurogenesis following stroke	5–42 days	IHC: DCX	Cells that express markers associated with neuroblasts are present in the ischemic penumbra surrounding cerebral cortical infarcts, where these cells are preferentially localized in the vicinity of blood vessels
Macas et al. (2006)	9 males, 3 females; aged 52–87	Ischemic stroke	Determine if stroke can elicit a response from endogenous neural progenitor cells in human brain	5 and 8 days, and 4 months	IHC: PSA-NCAM	Ischemic injuries increase generation of neural precursor cells in the SVZ. There is a decline in the number of neural precursor cells migrating along the olfactory tracts in older patients; nevertheless, the injury reactions to ischemia still occur despite the advanced age of the affected individuals

(continued)

Table 1.3 (continued)

References	Stroke patients	Stroke type/ location	Aims/objectives	Time post-stroke	Neurogenesis markers/ Technique	Results/findings
Marti-Fabregas et al. (2010)	7 patients with mean age 82	Ischemic stroke	Determine if neural precursor cells in the SVZ are activated after an ischemic stroke to repair the injured brain	Mean time of 10 days	IHC: PSA-NCAM GFAP-delta	Ischemic stroke increases number of migrating neuroblasts and immature neurons, indicating proliferation and migration to the site of ischemic lesion. Cells were not observed in the adjacent region of the SVZ due to the short survival time
Minger et al. (2007)	85-year-old patient	Ischemic stroke	Determine if there was evidence of altered endogenous neurogenesis in a patient who suffered a cerebrovascular accident 1 week prior to death	1 week	IHC: Nestin Musashi PSA-NCAM Sox2	Following ischemic stroke, there is increased endogenous neurogenesis which is associated with neovascularization and migration of newly formed cells toward a region of cerebrovascular damage in the adult human brain

Nakayama et al. (2010)	6 males, 4 females; aged 34–84	MCA ($n = 8$) ACA ($n = 4$) PCA ($n = 3$) BA ($n = 2$)	Examine the temporal and spatial expression of neural progenitor cells, in patients who suffered cardiogenic cerebral embolism	1–360 days	IHC: Nestin Musashi-1 βIII-tubulin CD31	Cardiogenic cerebral embolism causes regional activation of neural progenitor cells in affected human cerebral cortex during the acute to subacute period. There is little contribution of the latter cells to CNS repair at longer time points (90 days)
Stepien et al. (2018)	Ischemic stroke: 7 men, 7 women; mean age 70 ± 6.03. Hemorrhage stroke: 6 men, 2 women; mean age 64.8 ± 12.2. Control: 6 men, 2 women, mean age 64 ± 10.95	Ischemic stroke Hemorrhagic stroke	Identify and compare density of neurogenic cells between ischemic stroke patients and hemorrhage stroke patients with control patients	Ischemic stroke: 8 h – 78 days Hemorrhage stroke: 9 h – 22 days	IHC: GFAP-positive neural stem cells p-Histone H3Ser10-positive neural progenitor cells	GFAP-positive neural stem cells and cells labeled with p-Histone H3Ser-10 transcriptionally active cells of neurogenesis were present in both the DG and the SVZ of ischemic and hemorrhagic brains. Cells with p-Histone H3Ser-10 expression were significantly higher in DG following hemorrhage stroke when compared to ischemic stroke and control patients

Abbreviations: *IHC* immunohistochemistry, *h* hours, *DCX* doublecortin, *PSA-NCAM* polysialylated neuronal cell adhesion molecule, *SVZ* subventricular zone, *GFAP* glial fibrillary acidic protein, *ACA* anterior cerebral artery, *MCA* middle cerebral artery, *PCA* posterior cerebral artery, *BA* basilar artery, *DG* dentate gyrus

increased the proliferation of neural progenitor cells in the ipsilateral SVZ of stroke patients. Six weeks following stroke, these immature neurons have been shown to migrate to the region of cerebrovascular damage where they preferentially localized in the vicinity of blood vessels (Jin et al. 2006; Marti-Fabregas et al. 2010; Minger et al. 2007). Despite this proliferation of neuronal progenitors, one study reported that no surviving differentiated mature neurons were observed at 12 weeks after stroke in the previously ischemic region (Nakayama et al. 2010). Collectively, these results indicate that neural stem cells in the SVZ have the potential to play a crucial role in neuronal regeneration after cerebral ischemia.

1.5 Limitations Related to Endogenous Post-stroke Neurogenesis

There is extensive evidence that endogenous neurogenesis is important in functional recovery from stroke, but there are some limitations. One of the major limitations is the failure of post-stroke neurogenesis to produce adequate mature neurons to replenish those damaged or lost during stroke. Arvidsson et al. (2002) found that >80% of new neurons died during the first 2 weeks following stroke. The survival rate of adult neural progenitor cells following stroke is poor, and only a small percentage of the cells differentiate into mature neurons (Arvidsson et al. 2002; Bacigaluppi et al. 2008; Doeppner et al. 2011; Zhang et al. 2004b, 2011). Numerous factors contribute to this low survival rate, including the inflammatory response, high levels of oxidative stress, as well as a lack of neurotrophic support. Recent evidence suggests that activation of microglia and the subsequent release of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α create an inflammatory microenvironment that does not support survival of neural progenitors. For example, Ekdahl et al. (2003) demonstrated a negative correlation between the number of activated microglia in the SGZ and the number of surviving new hippocampal granule neurons. Furthermore, IL-6, a key inflammatory cytokine released by microglia, contributes to the reduction in neuron survival and neurogenesis (Monje et al. 2003; Vallieres et al. 2002).

The hostile conditions present in the brain post-stroke are not the only hurdles maturing neurons face. New neurons often fail to integrate into neuronal networks after stroke either due to incorrect morphology or signaling complications. Pathological or aberrant neurogenesis has been found to occur in 5–10% of new granule neurons after stroke. These neurons have morphological abnormalities including additional basal dendrites (meaning the cells appear to be bipolar) and ectopic cell position outside the granule cell layer (Niv et al. 2012). These misshapen neurons appear to be able to integrate into existing networks, but they and normal-appearing neurons exhibit altered dendritic complexity and spine morphology, indicating that their functionality may be altered (Niv et al. 2012). Indeed, one potential explanation for reduced cognitive function after stroke is due to newly matured neurons failing to functionally integrate. Cuartero et al. (2019) found that the number of immature neurons in the SGZ after stroke was inversely related to cognition (demonstrated

using the contextual fear conditioning test). While counterintuitive, this indicates that increased neurogenesis after stroke leads to more severe cognitive deficits. Such altered functionality of new neurons has been reported in other neurological injury models including epilepsy (Cho et al. 2015). If new neurons are unable to properly integrate into the existing neuronal networks, they will not be able to contribute to improved functional outcomes after stroke.

Finally, it is well understood that the rate of neurogenesis decreases with increasing age. In aged rodents, the proliferation rate of neuronal progenitor cells in the SGZ is decreased by 80% compared to young animals (Jin et al. 2003a; Kuhn et al. 1996). Interestingly, the proliferation rate of neural stem cells in the SVZ undergoes no significant (Kuhn et al. 1996) or a less severe (Jin et al. 2003a; Luo et al. 2006) decrease with age. This region-specific difference in neuronal progenitor proliferation was also observed in aged animals following stroke. Jin et al. (2004) showed that the aged rat brain is more sensitive than the adult rat brain to ischemic neuronal damage. The results revealed that stroke failed to stimulate neurogenesis in the SGZ, while ischemia-induced neurogenesis persists in the SVZ of aged rats (Jin et al. 2004). Furthermore, there were less granule neurons as well as basal neurogenesis in the SGZ of aged stroke animals compared to sham animals (Darsalia et al. 2005). Aged animals also had an impaired ability to differentiate newly formed neuroblasts into granule neurons in the dentate gyrus compared to young animals (Darsalia et al. 2005). These age-related decreases in post-stroke neurogenesis might be due to an intrinsic decline in neural stem cell responsiveness to stimulating environmental cues, decline in or disappearance of these environmental cues, and the appearance or accumulation of inhibitory factors. Therefore, interventions that can stimulate neurogenesis and promote the integration of new neurons leading to functional restoration would be highly desirable to promote post-stroke recovery.

1.6 Strategies to Promote Post-stroke Neurogenesis

Given that the majority of long-term disability in stroke stems from neuron loss, stimulating neurogenesis has long been viewed as a promising avenue for stroke therapeutics. As outlined above, although endogenous post-stroke neurogenesis does occur, there are several factors that limit neurogenesis sufficient to replenish lost neurons. Endogenous post-stroke neurogenesis needs to be enhanced to have a substantial impact on functional recovery after stroke. Therefore, strategies to inhibit inflammatory responses and to promote the release of neurotrophic factors have been suggested as effective in improving post-stroke neurogenesis (see Fig. 1.2). A few of the more promising therapies are briefly outlined below.

1.6.1 Pharmacological Interventions

Anti-inflammatory The post-stroke brain presents many challenges for neuronal maturation, one of which is the high levels of inflammation. Inflammation leads to

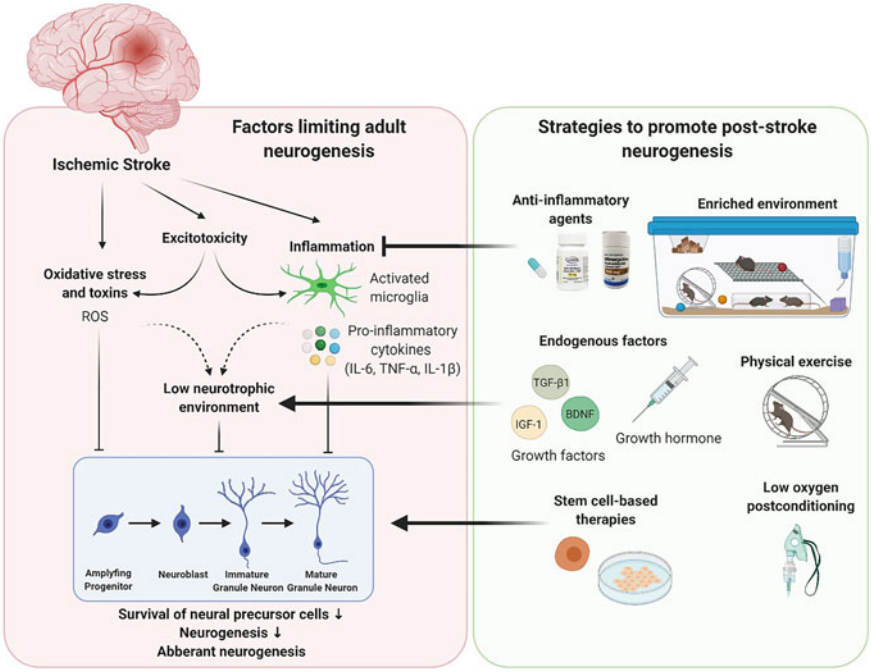

Fig. 1.2 Factors limiting post-stroke adult neurogenesis (red) and strategies to promote post-stroke adult neurogenesis (green). Following ischemic stroke, there are numerous factors that can contribute to disruption of neurogenic processes such as high levels of oxidative stress, activated inflammatory responses, and low neurotrophic support. Several pharmacological and non-pharmacological strategies to inhibit inflammatory responses and to promote the release of neurotrophic factors have been suggested as promising therapies to promote post-stroke neurogenesis. Abbreviations: ROS, reactive oxygen species; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor; TGF-β1, transforming growth factor-β1; IGF-1, insulin-like growth factor 1; BDNF, brain-derived neurotrophic factor

the generation of reactive oxygen species and the recruitment of inflammatory cells such as neutrophils, which are detrimental to neurogenesis (Tobin et al. 2014). Preclinical trials of anti-inflammatory pharmacological agents have yielded promising results. *Indomethacin*, a non-steroidal anti-inflammatory drug, increased the number of neuroblasts in the striatum after stroke in a rat model of transient middle cerebral artery occlusion (MCAO) as determined using BrdU labeling (Hoehn et al. 2005). In a similar preclinical trial, *minocycline* appeared to reduce microglial activation and led to a greater number of mature granule neurons in the dentate gyrus when compared to animals not exposed to minocycline (Liu et al. 2007). To demonstrate this, Sprague-Dawley rats were exposed to transient MCAO and given minocycline via intraperitoneal injection 4 weeks after stroke. Post-mortem analysis of brain tissue noted a twofold increase in BrdU+ neurons in rats exposed to minocycline. While the influence of anti-inflammatory therapeutics on neurogenesis is yet to be directly examined in humans, a recent review indicates that

only two clinical trials of anti-inflammatory treatment after stroke have demonstrated functional improvement, while the majority reported either no effect or worse outcomes for stroke patients (Tobin et al. 2014). Anti-inflammatory therapies should be viewed with caution as the inflammatory environment after stroke does serve a neuroprotective role as well as a destructive one.

Endogenous Factors Endogenous factors are particularly appealing as a pharmacological intervention after stroke due to their existing role in neurogenesis. *Growth factors* are one of the most studied neurogenesis-stimulating endogenous factors in stroke therapeutics due to their important role in proliferation and differentiation of numerous cell types, not just neurons. Given the importance of the neurovascular unit to normal neurological function, neurogenesis often needs to be accompanied by differentiation of complementary cells. Some growth factors that have been trialled preclinically include *transforming growth factor-β1 (TGF-β1)* (Ma et al. 2019) and *insulin-like growth factor 1 (IGF-1)* (Zhu et al. 2008). Ma et al. (2019) demonstrated that intranasal delivery of TGF-β1 increased the number of BrdU+ neurons and reduced infarct size after MCAO in mice. Zhu et al. (2008) virally transduced human IGF-1 into mice leading to overexpression of IGF-1. These mice were then exposed to MCAO and allowed to recover for 21 days. Mice carrying the IGF-1 transgene had a significantly smaller infarct and a greater number of BrdU+ neurons compared to empty viral vector controls. Similarly, *growth hormone* has been shown to stimulate neurogenesis and promote cognition. Ong et al. (2018) and Sanchez-Bezanilla et al. (2020a, b) have demonstrated in three separate studies that growth hormone promotes neurogenesis after experimental stroke. Specifically, they demonstrated that growth hormone treatment starting 48 h after experimental cortical stroke for 28 days promotes the expression of several growth factors (such as IGF-1 and VEGF) and proliferation of neural progenitor cells, as indexed by increased BrdU+ and DCX+ neurons within the peri-infarct region and the dentate gyrus. This proliferation was associated with the improvement of motor outcomes and cognitive function. Promisingly, small clinical studies have identified that growth hormone treatment ameliorates cognitive impairment in stroke patients (Feng et al. 2020; Song et al. 2012). Clinical trials of other growth factors have failed to replicate the exciting improvements noted in preclinical models (Lanfranconi et al. 2011). While growth factors have shown promise, it remains unclear why outcomes are so variable. One theory is that a single growth factor is not sufficient to direct the complex process of ensuring that new neurons differentiate, migrate, and integrate. Instead, a cocktail of substances may be required. Further trials are required to understand exactly which growth factors are the best to use, when to administer, and in what proportions.

Other endogenous factors, such as *sex hormones* and *neurotransmitters*, have been demonstrated to be capable of stimulating neurogenesis. Observational studies identified that women tend to have better outcomes after stroke than men, with a significant body of evidence indicating that this is due to the presence of sex-specific androgens (Haast et al. 2012). Upregulation of neurogenesis is known to occur in sync with the estrous cycle with the highest levels of proliferation occurring when

estrogen is at its highest concentration (Rummel et al. 2010; Tanapat et al. 1999). Neurotransmitters such as acetylcholine also show promise as therapeutic stimulators of neurogenesis (Biggio et al. 2009); however, these must be used carefully due to their potential impacts on homeostasis of the CNS.

Small Molecules The manufacture of small molecules that act as agonists or antagonists of receptors involved in the neurogenesis pathway has been one of the newest areas of neurogenesis stimulation research. One of the most promising molecules, *aminopropyl carbazole (P7C3-A20)*, increases nicotinamide adenine dinucleotide (NAD) flux in conditions that would normally cause cell death. In this way, P7C3-A20 modulates the environment to keep neural progenitor cells alive. In nonhuman primates, P7C3-A20 was capable of increasing the number of BrdU+ neurons (Bauman et al. 2018). When this compound was trialled by Loris et al. (2017) in rats exposed to MCAO, they found increased neurogenesis and reduced sensorimotor and cognitive deficits after stroke. The number of small molecules trialled to promote neurogenesis continues to grow exponentially. Time will tell if it yields a suitable therapy for stroke patients.

Stem Cell-Based Therapies *Stem cell and transdifferentiation* are two related forms of neurogenesis-stimulating therapy that have shown promise in preclinical models of stroke. Stem cell therapies involve the implantation of either endogenous or exogenous stem cells to supplement remaining neural progenitor cells, to encourage neuronal maturation, or to modify the environment to enhance the likelihood of maturation of neural progenitor cells. Liu et al. (2013) demonstrated that human embryonic stem cells are capable of differentiating into neurons that can successfully integrate into the hippocampus of mice in which neurons had been destroyed. Similar findings were seen in a rat permanent occlusion model where neural progenitor cells derived from human embryonic stem cells reduced infarct size and improved functional outcome (Kim et al. 2014). An ongoing issue with stem cell therapy is the risk of uncontrolled cellular proliferation and aberrant differentiation. Clinical trials that harness the power of stem cell therapy in stroke have taken place (Koh and Park 2017), but as yet none have led to a new stroke therapeutic. Transdifferentiation involves the differentiation of a somatic cell from one cell type to another. Niu et al. (2013) successfully modulated the transcription factor Sox2 in order to reprogram astrocytes into neuroblasts in vivo in the adult mouse brain. Further exposure of these neuroblasts to brain-derived neurotrophic factor (BDNF) and noggin stimulated maturation into functional neurons. As with stem cell therapies, transdifferentiation has numerous hurdles to overcome before it will be trialled in the clinic. For more information on the utility of stem cells for stroke recovery, refer to Chap. 10.

1.6.2 Non-pharmacological Interventions

Physical exercise has been shown to promote neuroplasticity after stroke in preclinical and clinical studies (Ploughman et al. 2015). Luo et al. (2007) reported that in adult mice, exposure to voluntary wheel running after ischemic stroke may promote the survival of immature neurons in the dentate gyrus by upregulating cAMP response element binding protein (CREB) phosphorylation. They also noted that animals exposed to voluntary exercise had improved hippocampus-dependent memory when compared to those not exposed to exercise after stroke. Aerobic exercise using a motorized treadmill for 30 min, once a day for 2 weeks, promoted the recovery of brain function by increased proliferation of BrdU+ and DCX+ neurons and BDNF expression, and a decrease in apoptosis after experimental cerebral ischemia (Seo et al. 2014). Nevertheless, the parameters of physical exercise, such as timing of exercise initiation, volume, intensity, and session frequency, should be carefully considered to avoid detrimental effects on functional outcomes after stroke (The AVERT Trial Collaboration group 2015).

A large body of evidence has demonstrated that an *enriched environment* and *brain training* can modify neurogenesis. Enriched environment is a complex combination in which spatial, visual, social, physical, and learning activities are tightly linked and interact together as an entire unit. In the context of stroke recovery, Komitova et al. (2005) demonstrated that enriched environment increased the number of neural progenitor cells in the adult rat SVZ, and these cells were recruited to the stroke injury at 5 weeks after a cortical stroke. Wurm et al. (2007) used a rat cortical infarct model to demonstrate that exposure to either rehabilitative training of the stroke-impaired forelimb or an enriched environment increased the number of BrdU+ neurons in the dentate gyrus and improved spatial learning. While preclinical studies have shown beneficial effects of enriched environment on stroke recovery, there are obvious differences when enriched environment is translated and implemented to the clinical setting (McDonald et al. 2018).

Intermittent exposure to a *low oxygen post conditioning (LOPC)* is an extremely promising, non-pharmacological therapy that has been shown to promote neurogenesis after stroke. Tsai et al. (2011) found that exposure of rats to LOPC post-stroke (12% O_2 for 4 h/day for 7 days) increased the number of BrdU+ neurons in the hippocampus compared with stroke animals not exposed to LOPC (Tsai et al. 2011). Furthermore, the authors used c-fos colocalization to demonstrate that the new neurons were more active in LOPC-treated animals. This increase in activity was positively correlated with improvements in cognition, indicating that they had functionally integrated into the existing network. A subsequent study by Tsai et al. (2013) expanded on these findings by double labeling with BrdU and DXC to show that the decrease in endogenous neurogenesis after stroke can be reversed using LOPC (Tsai et al. 2013). Together, these findings suggest that LOPC induces proliferation and enhances maturation of hippocampal neurons in rats. This non-pharmacological approach has a well-characterized and acceptable safety profile, is relatively low cost, and is easy to deliver and scale making it a very appealing potential therapy for clinical translation.

Dietary intervention is yet another non-pharmacological strategy for encouraging neurogenesis. The capacity of diet to influence adult neurogenesis has been thoroughly reviewed by Stangl and Thuret (2009), with flavonoids and curcumin supplementation, as well as caloric restriction demonstrating increased neuronal proliferation and increased survival, respectively. The recent Cognitive Ageing, Nutrition and Neurogenesis (CANN) trial set out to determine whether dietary supplementation with n-3 FLAV (docosahexaenoic acid + eicosapentaenoic acid + n-3 FLAV) was capable of attenuating cognitive function decline in a mild cognitive impairment population (Irvine et al. 2018). Outcomes from this trial are yet to be published. For more information on targeted dietary therapy for neurogenesis in stroke patients, refer to Chap. 10.

1.7 Concluding Remarks

There is robust evidence to support the existence of post-stroke neurogenesis in adult humans and animals. The ability of the brain to repair itself by endogenous neurogenesis after injury has led to numerous studies attempting to enhance it in order to improve outcomes for stroke patients. While post-stroke neurogenesis represents an attractive target for stroke recovery, there are caveats and fundamental issues that have prevented this promising therapeutic avenue from being implemented in the clinic. These hurdles can be overcome with a better understanding of neurogenesis, including the mechanisms of proliferation, differentiation, migration, survival, and functional integration. Pharmacological and non-pharmacological approaches to stimulate the generation of new, functionally integrated neurons have generated promising results. As with any translational efforts, the hurdles to the clinic are large. To improve the clinical viability of these strategies, future studies should incorporate aged animals, comorbidities (hypertension, diabetes), different times of delivery during stroke recovery (acute, subacute, and chronic), and combined approaches (such as physical exercise, enriched environment, and pharmacological therapy). With further research, enhancing post-stroke neurogenesis may be an extremely effective therapeutic option for improving outcomes and quality of life for stroke survivors.

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Application of Nanotechnology in Stroke Recovery

2

Reena Chittora and Suman Jain

Abstract

Stroke is a devastating condition, for which there is still no effective therapy. Nanotechnology is advanced and advantageous over conventional drug therapies due to its small size and programmed mechanism of action which specifically target the affected brain areas. By expanding novel advanced technology of tiny particles, scientists now aim to localize affected areas and attenuate the etiology of stroke. A variety of nanomaterials has shown beneficial effects in *in vitro* and *in vivo* stroke models and many more nanotechnology-based medicinal products are expected to flourish the stroke research in the near future. This chapter summarizes the latest advances in the application of nanoparticles, nanoscaffolds, nanomedicine, and nanocarriers in stroke.

Keywords

Stroke · Nanoparticles · Nanocarriers · Nanoscaffolds · Nanomedicine · Nanotherapy

2.1 Introduction

Stroke is the second leading cause of death and disability worldwide (Gorelick 2019). A report by Saver in 2006 stated that nearly 1.2 billion neurons, 8.3 trillion synapses, and 4470 miles or 7140 km of myelinated fibers are lost during a stroke episode. Following an ischemic attack, where there is a delay in receiving treatment, it is estimated that the number of neurons lost is equivalent to the number of neurons lost in 36 years of normal aging (Kyle and Saha 2014). Most stroke events are

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thromboembolic in nature and can be classified broadly into hemorrhagic (rupture of blood vessel) or ischemic (narrowing or obstruction of the vessels). About 85% of all strokes are ischemic strokes wherein blood supply to a specific brain area is blocked or compromised. This lack of blood supply to the brain comprises insufficiency of oxygen, reduced availability of nutrients, and inadequate removal of metabolic wastes leading to cellular toxicity, oxidative stress, inflammation, and cell death. This also results in excessive accumulation of fluid, which elevates intracranial pressure and manifestation of the clinical symptoms of stroke. Acute ischemic stroke is also associated with high concentrations of glutamate in the blood and interstitial brain fluid that exerts further excitotoxic effects on healthy tissue surrounding the infarct zone (del Carmen et al. 2013). Stroke symptoms include cognitive deficits, mood disorders, fatigue, apathy, anxiety and depression, and loss of muscular control often including slurring of speech (Hackett et al. 2014).

2.2 Pathophysiology

In stroke, cerebral blood vessel becomes obstructed which results in hypoperfusion, hypoxia, glucose deprivation, and ultimately neuronal death in the affected area. The circle of Willis functions as a safety valve for the brain, allowing collateral circulation to a compromised area. However, in stroke, it is often incomplete as the obstruction mainly occurs downstream, which limits the compensatory blood flow via the circle of Willis. Therefore, the center (core region) of the infarct zone gets hypoperfused severely and undergoes immediate necrosis, whereas, the surrounding region (penumbra region) is partially injured and undergoes apoptosis (Sarmah et al. 2020). Once ischemia ensues it initiates a vicious cycle of biochemical, metabolic, cellular, and vascular alterations. There is loss of ATP production by mitochondria, altered membrane permeability which changes the ionic milieu with gain of calcium and sodium ions, and loss of extracellular potassium ions. BBB disruption results in the infiltration of various inflammatory cells into the ischemic zone, releasing proinflammatory cytokines and generation of free radicals. Associated with these there is excessive release of excitatory neurotransmitters like glutamate that causes calcium-mediated excitotoxicity. All these events in turn further affect the mitochondria and nuclear signaling pathways culminating in neuronal death (Auriel 2009; Sarmah et al. 2017) (Fig. 2.1).

2.3 Current Strategies

Various therapeutic strategies developed for stroke basically aim for either refurbishment of cerebral flow and/or minimize the deleterious effects of microenvironment alterations on neurons (Auriel 2009). Treatment options for stroke are extremely limited and thus represent an area of unmet clinical need (Westendorp et al. 2011). Conventional therapies mainly focus on clot dissolution or reduce inflammation by employing chemotherapeutic agents such as clot bursting drugs,

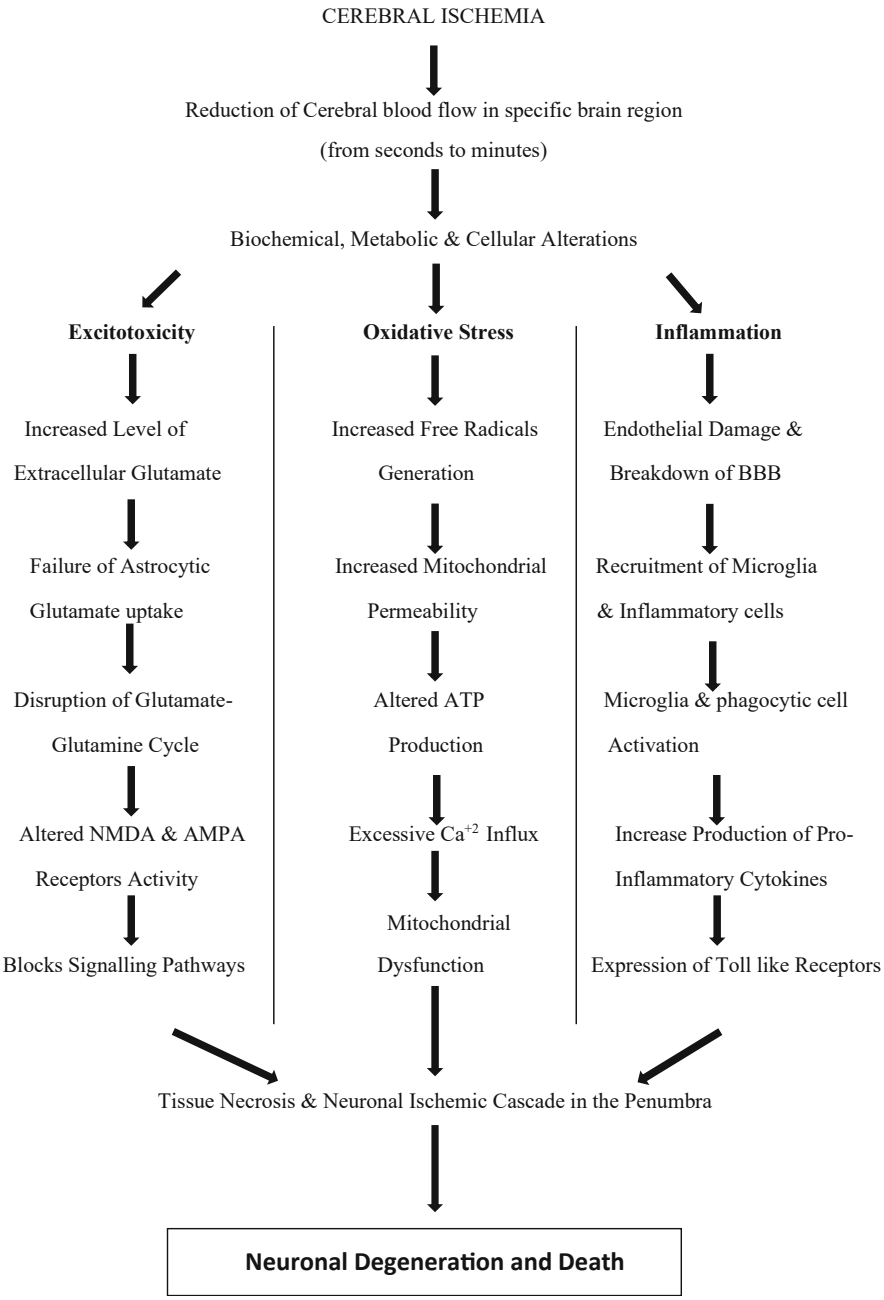

Fig. 2.1 Schematic diagram depicts pathophysiology of stroke

antiplatelet therapy, and neuroprotective drugs (Kaviarasi et al. 2019). Antioxidant therapy, such as Edaravone, is generally the first intravenous neuroprotective agent used for treating acute ischemic stroke (Nakase et al. 2011). Other protective and preventive therapies are oral anticoagulant/antiplatelet drugs to prevent stroke recurrence.

At present, thrombolysis and/or thrombectomy for restoration of blood flow are the only licensed treatments for ischemic stroke, but this can only be administered up to 4.5 h post-stroke and only a minority of patients are benefited with this treatment strategy (Tawil and Muir 2017). While reperfusion strategies are effective in opening up occluded cerebral vessels in few patients, there are currently no approved treatments for the inestimable damaging pathological processes such as oxidative stress and inflammation that persists for much longer durations after the acute stage (Wahlgren and Ahmed 2004). Therefore, targeting these downstream pathophysiological processes could hold great therapeutic potential.

2.4 The Need for Nanotechnology

The development of new therapies for stroke continues to face repeated translational failures due to insufficient concentrations of drugs that reach the intended target area (Sydserff et al. 2002). A major challenge in developing anti-stroke drugs is the complexity of the signaling processes involved as well as the associated inflammatory response and inaccessibility to the infarcted zone that further complicates the problem. Another important issue is the time window between the onset of stroke and treatment initiation which has been frequently wider in clinical trials compared to successful experimental stroke studies (Wahlgren and Ahmed 2004). Delivering drug molecules across blood-brain barrier, which is a highly selective permeable barrier between blood and brain, is yet another challenging task. In many blood-borne therapies brain endothelial cells form paracellular and transcellular barriers, so the development of efficient delivery strategies is highly warranted. Thus, developing new technologies that can dodge inefficient brain delivery and/or unfavorable distribution and safety profiles would lend new prospects to already existing therapeutics. To be able to devise novel treatment of cerebral ischemia, the identification of neuroprotective targets must be exclusive and limited to the neuronal injury area.

2.5 Nanotechnology

Regular drugs often get distributed all over the body and may affect all organs, so to reduce the side effects, in recent years emphasis has shifted to designing novel nanoparticles. They are “smart particles” because of the ability to sense and target ischemic brain regions only (Amani et al. 2019). Supporters promise to maneuver nanotechnology to spark a wave of revolutionary and novel products from machines to medicine. Nanotechnology, a new player in biomedicine, is contributing to neuroprotection by identifying hot-spots, disease mechanisms, and determining the

finest therapeutic windows. Nanotechnology is the ultrasmall scale technology typically from 1 to 100 nm, which has the ability to detect and modify the disease-causing processes at the molecular level. Nanoparticles provide large surface area to carry drugs or imaging markers, preferentially to sites with complementary biochemistry. Only nanoscale biomaterials have the ability to mimic the natural structure and topography of the biological milieu. This in turn substantively reduces unwanted side effects (Landowski et al. 2020). Targeted therapy by nano drug delivery systems has gained much attention to treat various diseases such as cancer in recent years. Nanoparticles deliver a smaller amount of the drug to target tissues more precisely with a controlled release, which can increase the shelf life of the drug. They also increase surface area to carry a high concentration of the drug, facilitating drug solubility and absorption ability (Liu et al. 2018a, b). Despite all the advantages, nanoparticles can be more toxic than large particles of the same composition because of these chemical properties.

2.6 Nanoparticles Against Stroke

In current medical research, nanotechnology is a rapidly developing field that offers promising future perceptions for the treatment of various CNS disorders. It involves several distinct areas of its application to basically address neural tissue injury including nano-enabled drug delivery across the blood-brain barrier, nano-imaging, biocompatible nanocomposites, and the most complex and advanced nanorobots, nanowires, and nanochips for nanotherapy (Kubinová and Syková 2010; Shcharbina et al. 2013).

Hordes of nanoparticles which are 15,000 times smaller than a pinhead may be able to deliver vital drugs to the brain, offering new optimism to patients in the early stages of a stroke. Nanoparticles can be divided into 2 types: “reversible” and “non-reversible.” Reversible nanoparticles include carriers such as liposomes and micelles that have a shorter circulating half-life, are less stable, and are used as targeted delivery agents. However, non-reversible nanoparticles can be delivered by virtually any route, intravenously, intranasally, or transdermally and such administration are clinically advantageous. It includes nanospheres, nanocapsules, dendrimers, nanotubes, nanorods, nanostructures, and mesoporous materials (Xu et al. 2014; Kemp et al. 2016). Most of these nanoparticles have been delivered through intravenous and intra-arterial routes, extending the restricted permeability of the BBB and the drug’s half-life (González-Nieto et al. 2020).

Various forms of nano-approaches are under experimental trials for recovery from stroke in *in vivo* or *in vitro* conditions. Broadly they can be classified as: metal nanoparticles, lipid-based nanocarriers, nanoscaffolds, and polymeric nanoparticles (Fig. 2.2).

Metal Nanoparticles Metallic nanoparticles have huge potential in nanotechnology because they can be synthesized and modified easily and can be conjugated with

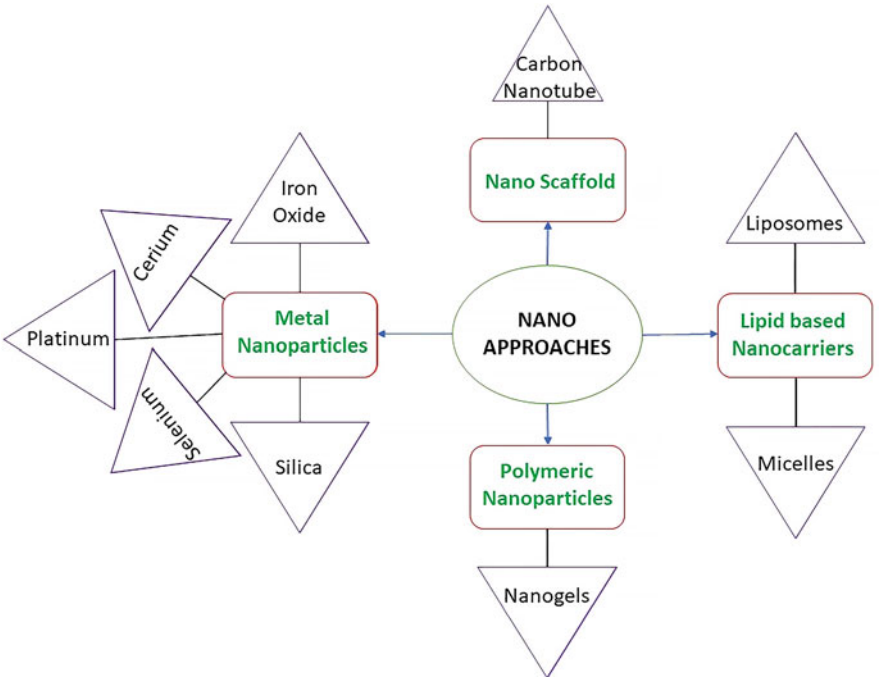

Fig. 2.2 Nano-approaches under preclinical testing for stroke therapy

the drug of interest, antibodies, ligands, etc. They have wide applications in diagnostics, imaging, and as a vehicle for gene and targeted drug delivery.

(a) Selenium Nanoparticles

Selenium (Se) nanoparticles have emerged as a promising tool for combating major CNS disorders. It is an essential trace element, which has unique physiological and pharmacological properties to attenuate the incidence of neurodegenerative diseases (Burk et al. 2014; Yang et al. 2017). In the ischemic stroke murine model, selenium nanoparticles exhibit high antioxidant activity that reduces oxidative stress and related cell death etiology due to stroke. The nanoparticles affect the metabolism of nerve cells and suppress inflammation which is a major culprit of the harmful effects of stroke. Administration of the biodegradable selenium nanoparticles in the murine model also leads to resolution of brain edema, myelination, and protection of hippocampal axons after cerebral ischemic stroke, ensuring efficient targeting with minimal side effects. Amani et al. (2019) were the first to synthesize Se NPs (with sizes ~12 nm) and study *in-vivo* targeted therapy in the brain of the ischemic stroke rat model. This study provided a promising treatment strategy based on anti-transferrin receptor monoclonal antibody (OX26)-PEGylated Se nanoparticles (OX26-PEG-SeNP), as it significantly induced suppression of

excessive inflammation and oxidative stress through modulation of signaling pathways. The seleno-proteins are also significantly expressed in the human brain through dietary Se intake, and their characteristic antioxidant activity contributes to the free radical defense system. Furthermore, Se administration contributes to mitophagy of impaired mitochondria generated after focal cerebral ischemia (Mehta et al. 2012; Wadhwani et al. 2016).

(b) Ceria Nanoparticles

Reactive oxygen species-induced oxidative damage is one of the most critical mechanisms responsible for causing ischemic injury. Ceria nanoparticles exhibit superoxide dismutase and catalase mimetic activity that protect cells against two dominant ROS, the superoxide anion and hydrogen peroxide (Singh et al. 2011). A study from an *in vivo* rodent model depicted that an optimal dose of ceria nanoparticles (0.5–0.7 mg/kg) has powerful neuroprotective effects against ischemic stroke (Kim et al. 2012). Cerium oxide (CeO_2) nanoparticles (3 nm in diameter) were tested in rat tMCAO models for their radical-scavenging activities when coated with PEGylated phospholipids (30 nm). There was a dose-dependent increase in the concentration of Ce nanoparticles in the ischemic hemisphere as compared to non-ischemic hemisphere of the rat brain following stroke. Further, a moderate dose of 0.5 and 0.7 mg/kg was able to significantly reduce oxidative stress, infarct volume, number of apoptotic cells, and levels of pro-apoptotic proteins *in vivo*, whereas, low (0.1 and 0.3 mg/kg) and high (1 and 1.5 mg/kg) doses did not exhibit any neuroprotective effects (Kim et al. 2012).

(c) Iron Oxide Nanoparticles

In stroke, iron oxide nanoparticles after nasal administration were found in high concentrations in the hippocampus and striatum which are sensitive to ischemic injury. Superparamagnetic iron oxide (SPION) nanoparticles have the capability to cross the BBB and are thereby used in MRI as a contrast agent for evaluating stroke size and location as well as a high-resolution assessment of the extent of injury. SPION Ferumoxytol (Feraheme), carboxymethyl dextran-coated iron oxide is currently FDA-approved for imaging (Landowski et al. 2020). PEG-coated superparamagnetic iron oxide nanoparticles help in identifying the BBB compromised area by colocalizing with Gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA) (which is a marker of BBB injury) during imaging (Liu et al. 2014). In animal models of brain ischemia, for the detection of early inflammatory responses, iron oxide nanoparticles are used, as they are selective markers for P-selectin and other adhesion molecules (Da Silva-Candal et al. 2017). Nanocubes made up of superparamagnetic iron oxide particles coated with tPA, when excited with an alternating magnetic field, accelerated lysis of thrombi by creating local hyperthermia (mechanical lysis) as well as by interaction of tPA with the fibrin network (chemical lysis) (Voros et al. 2015). These nanocubes were also shown to

have high transverse relaxivity, thereby suggesting them to be a potent therapeutic agent for thrombolytic disorders.

(d) Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles have manageable, well-defined microstructure (large surface area and pore volume), greater capacity for drug loading, and surface functionalization with biocompatibility which attract them for drug delivery. These nanoparticles are able to cross the blood-brain barrier and also have biodegradable polymer coating for controlled drug release (Shen et al. 2018).

Cha et al. (2018) recently reported in the rodent model of intracerebral hemorrhage (ICH) that lipid-coated magnetic mesoporous silica nanoparticles loaded with ceria nanoparticles (LMCs) have the property of scavenging reactive oxygen species and thereby exhibit strong antioxidative and anti-inflammatory activity in stroke.

(e) Platinum Nanoparticles

Platinum nanoparticles (nPt) have large surface area and high electron density which make them potential and novel ROS scavengers. In the transient middle cerebral artery occlusion (tMCAO) mouse model, nPt of 2–3 nm size ameliorated superoxide free radical generation and decreased infarct volume in ischemic/reperfusion injury. nPt also inhibited the MMP-9 activity and ameliorate the disrupted neurovascular unit (NVU) after stroke (Takamiya et al. 2012; Dong et al. 2020), suggesting a potential therapeutic role in stroke.

2.7 Polymeric Nanoparticles

Polymeric nanoparticles (PNPs) are biodegradable, highly biocompatible, nontoxic by-products, widely used as the drug delivery systems for oligonucleotides, proteins, and small molecules for the treatment of ischemic stroke. PNPs are administered intravenously that target delivery of platelets or fibrins to stabilize structure and reduce bleeding time in the hemorrhagic stroke mouse model (Bertram et al. 2009). PNPs are also used to deliver antioxidants and neuroprotective agents. Delivery of TNF-α to the infarct site in a rat model of ischemia/reperfusion-induced cerebral injury using a polyelectrolyte complex formed with the biocompatible diblock copolymer (poly(ethyleneglycol)-b-(poly-ethylenediamine-Lglutamate-g-poly (L-lysine) ameliorated apoptosis and inflammatory activity (Xu et al. 2017). For treatment of post-ischemic complications, si-RNA loaded in polyplexes PEGylated poly(L-lactide) nanoparticles incorporated in mice models of cerebral ischemia-reperfusion injury showed silencing of C3 in microglia (Wang et al. 2018). Antioxidant enzyme SOD-loaded polymeric poly (lactic-co-glycolic acid) (PLGA) nanoparticles injected via the intracarotid route in a middle cerebral artery occlusion (MCAO) rat model reduced infarct volume by 65% in comparison to 25% reduction

observed when free SOD alone is given. SOD-nanoparticle application resulted in 75% survival (Reddy and Labhasetwar 2009).

(a) Nanogels

Nanogels are nanoscale, three-dimensional, cross-linked polymers that act as reservoirs from which drugs are released. They offer the benefit of controlled release; however, their surface area may be greater due to the amorphous structure and high water content. They are innately soft compared to other drug delivery systems and have a greater capacity of loading therapeutics, which may be suspended in the nanogel or form part of the structure (Alkaff et al. 2020). Studies in rat pMCAO models of thrombolysis had used a PEGylated thrombolytic agent, urokinase (UK), as the nanogel polymer that dissociated in low pH of the ischemic microenvironment. In yet another study UK was loaded onto chitosan nanogel that dissociated upon ultrasound stimulation (Cui et al. 2016; Teng et al. 2018).

2.8 Nanocarriers

To enhance thrombolysis in ischemic stroke and to target the 2 main components of blood clots, i.e., fibrin or platelets, different types of nanocarriers are used to vectorize plasminogen activators (PAs) to the thrombus site (Liu et al. 2018a, b). Despite the great potential in the treatment of stroke, nerve growth factor (NGF) is not able to pass through the blood-brain barrier which makes clinical administration dependent on invasive neurosurgical procedures (Bonner and Peskind 2002). Exploring the bio-distribution of nanocarriers is an important requirement for clinical translation of nanocarrier delivery (Feczkó et al. 2019).

(a) Albumin Nanocarriers

Xu et al. (2018) prepared resveratrol-loaded human serum albumin nanoparticles (RES-HSA-NPs) to facilitate protection from cerebral ischemic/reperfusion injury by RES. RES-HSA-NPs of spherical shape with a diameter of about 100 nm and dose highest RES encapsulation (dose 20 mg/kg) when injected intravenously into transient middle cerebral artery occlusion (tMCAO) rats, improved neurological score and decreased infarct volume after 24 h and 72 h by significantly attenuating oxidative stress and neuronal apoptosis (Xu et al. 2018).

(b) Theranostic Neuronal Growth factor (NGF)-loaded nanocarriers

NGF-loaded nanocarriers were manufactured by embedding ultra-small superparamagnetic iron oxide (USPIO) and NGF into human serum albumin (HSA) core particles followed by surface modification with ApoE. The nanoparticles of a similar size range (200–250 nm) were injected after inducing stroke in male Wistar rats by transient middle cerebral artery occlusion model. It revealed a

significant reduction of the infarct size compared to the vehicle control (Feczkó et al. 2019). In this approach, nanocarriers for the treatment of stroke comprising a biodegradable matrix of HSA and NGF were modified on their surface with ApoE in order to deliver the growth factor to the brain.

Lipid-Based Organic Nanocarriers Lipid-based nanocarriers are easy to synthesize, are biocompatible, and have potential of encapsulating multiple functional substances with controlled release of therapeutic agents, making them attractive for biomedical use. Lipid core nanocarriers are used for both diagnosis and treatment of diseases and are high-capacity reservoirs for lipophilic drug entrapment.

(a) Liposome

Liposomes are made from lipids which are long chains of organic molecules found in all living organisms. Liposomes are used as a drug delivery system because of their good biocompatibility, biodegradability, blood-brain barrier permeability, and low toxicity (Bonnard et al. 2019). Research from the University of Manchester revealed that tiny vesicles (just 100 nanometers in diameter) called liposomes can translocate through the damaged blood-brain barrier following stroke which may offer a potential way to get vital drugs to the lesion sites to stop further damage. Liposomes were first used for the delivery of hemoglobin (Hb) in the stroke rat model that resulted in higher oxygenation at the infarcted site and reduced infarct sizes in the treatment group as compared to control (Kawaguchi et al. 2007; Fukumoto et al. 2009). In a global transient ischemic rat model, behavioral data showed that liposomal Hb improved cognitive impairment as well as motor function deficits (Komatsu et al. 2007; Hamadate et al. 2010). Cross-validation studies of liposomal Hb were carried out on monkeys, which showed that the infarct sizes were decreased as well as weight and muscle strength were regained in the treated group significantly as compared to controls (Kawaguchi et al. 2013). A recent study revealed that intravenous administration of liposomes recovered different phases of BBB disruption after stroke in mice exposed to transient middle cerebral artery occlusion at early (0.5 h and 4 h) and delayed (24 h and 48 h) time points (Al-Ahmady et al. 2019). Maintaining long-term liposomal co-localization within the neurovascular unit has great potential for neuroprotection. *In vivo* real-time imaging and histological analysis showed enhanced levels of liposomal uptake by glial cells late after experimental stroke (2–3 days) which highlights glial cells' potential for blocking delayed inflammatory responses and/or the role of liposomes in shifting the polarization of microglia/macrophages toward brain repair. This also suggests capability of liposomes to maximize selective translocation into the brain after stroke (Al-Ahmady et al. 2019). PEGylated liposomes loaded with citicoline (therapeutic agent) and conjugated with heat shock protein 72 (HSP72) antibody (a target protein that is specifically expressed in the peri-infarct ischemic region) selectively accumulate in the brain ischemic region and enhance the beneficial effect of the drug in ischemic middle cerebral artery occlusion male Sprague-Dawley rat model (Agulla et al. 2014). Peng et al. (2013) administered liposomes loaded with

Xenon gas (optimal dosage of 7–14 mg/kg) to rats for up to 5 h after stroke onset. It resulted in diminished infarct size due to Xenon's neuroprotective properties and crossing of the BBB by liposomes.

(b) Micelles

Micelles comprise amphiphilic molecules that self-assemble around a hydrophobic core to form colloidal spheres below 100 nm in diameter (around 5–50 nm). An individual molecular building block of a micelle may be monomeric, peptidic, or co-polymeric. Drugs may be conjugated to the surface of individual molecules or encapsulated in the core (Alkaff et al. 2020). Many antioxidants and anti-inflammatory compounds such as superoxide dismutase, curcumin, luteolin, puerarin, and resveratrol have been delivered in association with micelles to treat ischemic/reperfusion injury and post-ischemic inflammation (Tan et al. 2018; Mukherjee et al. 2019; Wang et al. 2019). An alternative strategy to modulate intracellular signaling involves targeting specific ligands to receptors, such as the channel blockers (MRZ2/576 and riluzole) as well as the neuropeptides or nerve growth factor (NGF) (Feczkó et al. 2019). R3V6 micelles (an example of a peptidic unit that comprises three blocks each of arginine and valine) were loaded with heme oxygenase-1 (HO-1) plasmid for delivery along with dexamethasone. It reduced lesion size and inflammation markers as compared to untreated controls and also served to increase both core stability and transfection efficiency (Lee et al. 2012). A study in tMCAO mice incorporated a thrombin-cleavable peptide sequence into a copolymer, to which then a ligand was conjugated to the surface for targeting to ischemic tissue. These micelles were loaded with glyburide and showed a significant reduction of cerebral edema and infarct sizes, as well as improvement of neurological scores (Guo et al. 2018). The intranasal administration of micelle-clonazepam induced a sustained release of the drug after approximately 30 min in comparison to intravenous administration (González-Nieto et al. 2020), suggesting differential effects of the drug depending upon the route of administration.

Radical containing nanoparticle (RNP), a novel core-shell-type nanoparticle consisting of micelles containing 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO), an antioxidant nitroxyl radical, was injected in MCAO male Sprague-Dawley rat model. RNPs scavenged free radical superoxide anions produced by ischemia-reperfusion injured neuronal cells and also decreased the cerebral infarction volume and improved neurological deficits after cerebral ischemia-reperfusion injury (Marushima et al. 2011). Hosoo et al. (2017), in transient middle cerebral artery occlusion C57BL/6J mice model, incorporated nitroxide radical-containing nanoparticles (9 mg/kg) through the common carotid artery after cerebral ischemia-reperfusion injury, which reduced blood-brain barrier damage and infarction volume by scavenging free radicals.

2.9 NanoScaffolds

Nanoscaffolds are fabricated, biodegradable, porous, biopolymer nanofibers which provide a suitable microenvironment for cell-cell interaction. It also induces cell attachment to extracellular matrix, cell proliferation, and differentiation, thereby facilitating delivery of drugs or materials to specific areas or nuclei.

Carbon Nanotubes

Another class of materials discovered for targeted drug delivery, on the basis of potential biocompatibility, purity, high aspect ratio, surface properties, and nanofluid nature are carbon-based nanomaterials. Carbon nanotubes (CNTs) are novel nanoscale structures (scaffolds) for drug delivery because of their ability to penetrate and deliver materials specifically to the damaged cells (Nair et al. 2012; Sarmah et al. 2020). Amine-modified single-walled carbon nanotubes (SWNTs) can reduce apoptosis, inflammation, and glial cell activation to protect the brain from ischemic damage (Lee et al. 2011). The attractive properties of CNTs, such as an increase in differentiation of neural stem cells to neurons and inactivation of macrophage, promise their use as novel stem cell delivery vehicles for treating stroke. The first evidence that CNTs can improve stem cell differentiation was reported in an *in vivo* study of focal cerebral ischemia male Sprague-Dawley rat model. Hydrophobic (HP) carbon nanotubes impregnated with subventricular zone neural progenitor cells (SVZ NPCs) reduced infarct cyst volume and cyst area after stroke (Moon et al. 2012). Another formulation of dexamethasone (glucocorticoid with anti-inflammatory properties) loaded, PEGylated (enhanced the release of dexamethasone), vertically aligned multiwalled carbon nanotubes (VA-MWCNTs) is reported as a potential ischemic stroke intervention (Komane et al. 2018). An *in vitro* study on aminated multi-walled CNTs incorporated with nerve growth factor (MWCNTs-NGF) showed increased cell viability in ischemic stroke PC12 cell lines (Hassanzadeh et al. 2017).

2.10 Nanomedicine

Various treatment strategies presently developed for providing neuroprotection after ischemic injury and treating stroke are not efficient because of delayed initiation of therapy. Therefore, pretreatment strategies before the onset of ischemia with neuroprotective drugs and herbal preventive drugs with lesser-known side effects are more crucial (Poellmann et al. 2018). Nanomedicine is a novel approach for efficient drug/herbal formulation delivery by enhancing its site-specific efficacy by crossing the BBB.

Perfluorocarbon (PFC) nanoparticles (artificial blood substitutes), which are lipid encapsulated NPs of 250 nm diameter, have been used for targeted drug delivery. *In vitro* study suggested that PFC thrombolytic nanoparticles target specifically fibrin fibers present in clots and cause their rapid dissolution (Marsh et al. 2011).

Silymarin, a bioactive constituent isolated from milk thistle, has antioxidative and tissue-regenerating properties. It was encapsulated inside a collagen-based polymeric nanoparticulates (nanosilymarin) of 48 nm size and injected intraperitoneally for 7 days at different doses (10, 100, and 1000 μg/kg). Nanosilymarin-treated focal cerebral ischemic rats showed remarkable improvement in neuronal loss, motor activity, reduction in infarct volume, and enhanced neuroprotection by antioxidative mechanisms at very low drug dosage (Rathore et al. 2020).

I. Oral Nanomedicine

Natural products or compounds are pleiotropic antioxidants with multiple medicinal values and have been in use since ancient times. They have low bioavailability, water insolubility, and cannot cross the BBB, that's why major stumbling block in stroke treatment and other CNS disorders. Nanoparticles between the sizes of 10 and 100 nm cannot be used as nanomedicine because they are not absorbed via the gastrointestinal tract. So, the development of nanotechnology-based medicines that can get absorbed in blood would be an ideal oral medication for stroke (Mutoh et al. 2016; Alavian and Shams 2020).

(a) Curcumin Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) have therapeutic potential for stroke treatment because they are lipophilic colloidal carriers with large surface area and high loading properties and have ability to penetrate the BBB. In study curcumin (25–50 mg/kg)-loaded solid lipid nanoparticles (C-SLNs; average particle size 134.6–15.4 nm) were administered orally in a bilateral common carotid artery occlusion rat model for 5 days before and 3 days after stroke induction. They reported that oral curcumin-SLNs improved the cognitive symptoms and provide neuroprotection by increasing superoxide dismutase, catalase, glutathione, mitochondrial complex enzyme activities in cerebral ischemia, whereas, oral curcumin alone could not cross the BBB and was found to be ineffective (Kakkar et al. 2013).

(b) Polylactide-co-glycolide (PLGA)-Encapsulated Quercetin

Ghosh et al. (2013) orally administered PLGA-encapsulated quercetin (2.7 mg/kg; 20–50 nm) 2 h before ischemic insult and until post-stroke day 3 in common carotid artery occlusion rat model. They evaluated the therapeutic efficacy of polymeric nanoparticulated quercetin which is a flavonol, the main ingredient of red and orange pigments in fruits and vegetables. It revealed strong antioxidative property, promoted neuronal survival by inhibiting caspase-3 activity, and also controlled altered osmolality observed in various brain regions following induction of stroke.

(c) Panax Notoginsenoside (PNS) Loaded Core-Shell Hybrid Liposomal Vesicles (HLV)

Core-shell hybrid liposomal vesicles (HLV) are novel liposomal-based polymeric nanoparticles (methyl ether PEG-PLGA-based) which increase entrapment efficiency and retard destruction of drug components for oral application. Panax notoginsenoside (PNS) is the main active ingredient derived from the root of the traditional Chinese herb Panax notoginseng (PN), which has various anti-inflammatory, anti-oxidative, vasodilator, and protective effects. When PNS-loaded core-shell HLV (PNS-HLV; 337.8–40.2 nm) was administered orally (30 mg/kg) for 10 days in Wistar rats before the induction of ischemia, it attenuated the ischemia/reperfusion-induced brain infarction. The encapsulated drug PNS in HLV exhibits greater effectiveness, higher potency, and a high concentration of drugs delivery into the circulation, thereby helping in attenuation of brain damage (Alavian and Shams 2020).

(d) Puerarin Hydroxypropyl beta Cyclodextrin (PUE-HP-β-CD)

Puerarin is an isoflavone found in herbal medicine and has been used in China for ischemic stroke treatment because of its antioxidative, antiapoptotic, and anti-inflammatory properties. But its poor bioavailability and low concentration in the brain restrict its clinical use. Hydroxypropyl beta cyclodextrin (HP-β-CD), a polymeric nanoparticle containing Puerarin (100–300 nm) when administered orally in rat ischemic stroke model, enhanced the penetration of the drug across the BBB and resulted in significantly lower infarct size, neuronal cell death 3 and 7 days after treatment (Tao et al. 2013).

II. Intranasal Nanomedicine

Intranasal drug delivery is a fast-acting treatment strategy because the olfactory neuro-epithelium is not protected by the BBB and due to their small diameter, the NPs easily cross the nasal membrane and are transported to brain through various endocytic pathways.

(a) Rutin-encapsulated Chitosan Nanoparticles (RUT-CS-NPs)

Rutin (RUT) is a flavonoid glycoside that is found in certain herbs and fruits, has various beneficial properties, such as antioxidant, anti-inflammatory, and neuroprotection, improves blood circulation, and prevents the formation of blood clots. Nanoparticles formulated by Chitosan (a biocompatible natural polysaccharide) incorporated with RUT when administered intranasally in brain ischemia rat model, it significantly decreased infarct size and improved behavioral outcomes in comparison to intravenous administration. Hence it is concluded that RUT-CS-NP is a safe, non-invasive, and effective drug delivery system for the ischemic brain (Ahmad et al. 2016).

(b) Polymeric N-isopropyl Acrylamide (PNIPAM) Nanoparticles of Curcumin (CUR), Demethoxycurcumin (DMC), and Bisdemethoxycurcumin (BDMC)

Curcumin, a natural polyphenol is well known for its antioxidative, antiseptic, and neuroprotective properties and has been in use in traditional medicine, but due to its poor bioavailability, rapid metabolism, rapid systemic elimination, and low absorption has not been confirmed as a therapeutic agent. In an *in vivo* experiment, MCAO rats were pretreated intranasally with PNIPAM nanoparticles of CUR, BDMC, and DMC (different components of *Curcuma longa*). The nanoformulation mainly PNIPAM-CUR showed high drug loading with the gradual release. It prevented ischemic injury, ameliorated behavioral changes, and oxidative stress in comparison to PNIPAM-DMC and PNIPAM-BDMC (Ahmad et al. 2013).

(c) 6-Gingerol-Loaded Mucoadhesive Nanoemulsion (GRL-MNE)

6-Gingerol (GRL) is a phenolic compound of ginger that has antioxidative and neuroprotective properties. Mucoadhesive nanoemulsion formulation for intranasal delivery of GRL to the brain in an ischemia rat model showed significant improvement in neurobehavioral and histopathological assessment as well as reduction in infarct volume at very low doses of GRL (Ahmad et al. 2020c).

(d) Glycyrrhizic Acid Encapsulated-Chitosan-Coated-PCL [poly(ε-caprolactone)] Nanoparticles

Glycyrrhizic acid (GRA), a triterpene saponin glycoside, is an antioxidant with neuroprotective properties and has been used as a flavoring agent and health remedies for thousands of years. *In situ* nasal gel formulation using polymeric nanoparticle and mucoadhesive agent, Chitosan (increase absorption and contact time with nasal mucosa), was prepared in a recent study for the treatment of cerebral ischemia. In this study novel formulation GRA-loaded-CS-coated PCL-NPs or surface-decorated nanoparticles administered intranasally in Wistar rats enhanced the drug bioavailability in the brain and helped to treat cerebral ischemia effectively (Ahmad et al. 2020b).

(e) Poloxamer-Chitosan-based Naringenin Nanoformulation (NRG-NE-gel+0.50% CS)

Naringenin, a natural flavonoid compound present in grapefruits and tomatoes, has anti-inflammatory and antioxidative properties. To improve the clinical therapeutic application of NRG, an innovative lipid-based intranasal drug delivery system is designed. *In situ* nanoemulsion is formulated using Chitosan (0.50% CS), mucoadhesive agent and poloxamer (20%), gelling agent for intranasal delivery of Naringenin. It led to significant drug delivery to the brain with improved retention time and mucoadhesive properties in the treatment of cerebral ischemia (Ahmad et al. 2020a).

2.11 Nanotherapy

Thrombolysis therapy is the touchstone treatment with intravenous administration of tPA (tissue-type plasminogen activator) for acute ischemic stroke (Powers et al. 2018). tPA converts the endogenous plasminogen into plasmin, which in turn lyses the fibrin mesh of the thrombi, thereby promoting recanalization or restoring blood flow. However, beyond the 3–4.5 h therapeutic window, tPA is associated with an increased risk of hemorrhagic transformation and the detrimental side effects outweigh the beneficial effects (Bonnard et al. 2019). A new approach to thrombolysis therapy is the use of nanoparticles coated with tPA and fucoidan, that can lyse the platelet-rich thrombi (Juenet et al. 2018). Various smart delivery nanodevices loaded with fibrinolytic drugs have been developed, which are triggered to release plasminogen activator by thrombus itself or by high shear stress caused by thrombosis or oxidative stress (Bonnard et al. 2019).

Even though a significant number of studies have shown improvement of morphological and functional deficits following stroke with various nano-approaches in preclinical research, none of these systems has yet been translated for patients' benefit. One of the reasons could be that the use of nanomaterials comes along with a substantial risk of toxicity when exposed to the tissues *in vivo*.

2.12 Safety and Limitations

Nanoparticles seem to be safe but some can exhibit toxicity or activate innate immune sensors when injected intravenously or intrathecally. Exposure to environmentally sourced nanosized particulates aggravates stroke in mice (Liu et al. 2016). Carbon nanotubes are morphologically similar to asbestos, thus can induce mesothelioma. Nanoparticles (50–60 nm size) injected intravenously which comprised of pure copper or silver and to a lesser extent aluminum increased blood-brain barrier (BBB) permeability which could be harmful in long run during stroke, as it may cause infiltration of other blood cells or proteins into the brain (Sharma et al. 2010; Haley and Lawrence 2017). It may also cause reentry of nanoparticles back into the systemic vasculature thereby limiting its efficacy in the brain. The major challenge is the lack of appropriate cytotoxic assessments done in parallel with functional studies on the fabricated nanomaterials.

2.13 Summary

The chapter highlighted various recent developments in Nanotechnology and Nanomedicine in preclinical studies of stroke. Stroke is a complex disease, whose recovery is dependent upon several factors like degree of damage, integrity of BBB, and therapeutic window of treatment. Nanotechnology has been able to offer a plethora of products that take care of these limitations and provide significant beneficial effects. However, to be able to translate this knowledge from laboratory

to bedside there is a need to develop careful safety screening protocols that can assess targeted drug delivery, efficacy, dosing, type of neural cells, time and route of administration, and toxicity, if any. Further, a conceptual understanding of biological responses to these nanomaterials is also required before the vectorization of molecules of interest with minimal side effects. Stuart Allan, Professor of Neuroscience from The University of Manchester stated: "The discovery that nanomaterials may be able to facilitate the treatment of stroke is exciting: scientists have long been grappling with the difficulties of treating brain injuries and diseases. The brain-blood barrier is a major frontier in neurology, so the prospect of being able to cross, it may have applications to other conditions as well – though clearly, much more work needs to be done." Though stroke nanotechnology offers a number of challenges and limitations, these discoveries are important milestones which pave the path for better stroke management in the future.

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3 Nanotechnology: A Daydream for Advanced Imaging, Diagnosis, and Therapeutic Approach for Cerebral Ischemia

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Abstract

Cerebral ischemia is one of the many deadly disorders that is caused due to the blocking of blood vasculatures in the brain and causing oxygen depletion thereby hampering the functions of the brain and can lead to permanent damage and death. Although a wide variety of elements can lead to cerebral ischemia, it is frequently considered as one of the old age-related illnesses. However, unhealthy dietary practices, stress-related factors, and degenerative environmental qualities have drastically lowered the onset age and number of cases in the past years. Hence, there arises an urgent necessity for the quick and efficient strategies for emergency therapy and chronic management of brain functions, as well as comparatively earlier and precise diagnosis of stroke-related events and an assessment of the extent of damage after the stroke occurrence. Pathophysiological complications further hamper the formulation of diagnostic and treatment strategies in cerebral stroke management. The advancement in nanotechnology has started newer paradigms for more effective and superior therapies and imaging techniques for managing cerebral ischemic disorders. This chapter gives briefings about the recent knowledge underlying the mechanisms and features of cerebral ischemia, limitations of traditional and presently existing

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therapies, and some novel targets which can be exploited for stroke management. It also provides a detailed discussion on the advancements achieved in the applications of nanotechnology-based approaches and technologies for therapeutic and imaging purposes in stroke along with their some advantages and lacunas. Emergent multifunctional and customized nanocarriers for stroke management have also been discussed.

Keywords

Ischemic stroke · Nanoparticles · Neuroprotection · Bioimaging · Nanotechnology

3.1 Introduction

Dysregulation of the fibrinolysis cycle obstructs blood flow to the brain which leads to stroke, which is a fatal condition and damages the brain cells. Due to this, specific body functions get hampered which is controlled by the stroke-affected region of the brain (Zhu et al. 2018). The condition of stroke depends on the extent of damage caused in the area of the brain and the interval for the restoration of the flow of blood. Therefore, stroke is classified into three categories depending upon the extent of damage and their root cause, namely, ischemic stroke, hemorrhagic stroke, and transient ischemic attack. Since ischemic stroke is instigated due to the obstruction in blood flow due to some kinds of blocks, whereas hemorrhagic stroke is caused due to the burst of any blood vessel, thereby resulting in leakage of blood. Lastly, the transient ischemic attack arises due to the development of minor embolism of clots that blocks the blood flow for some time. Nevertheless, the severity of damage caused by transient ischemic attacks is less as compared to the other two types of strokes; therefore, it divulges plausible jeopardy of more lethal and severe ischemic or hemorrhagic strokes (Duca and Jagoda 2016).

Cerebral strokes are considered the second most common cause of death around the world just after cardiovascular problems; the statistics say the mortality caused by ischemic stroke is ~ 87% (American Stroke Association 2020). The data of WHO exhibits that almost 1.65 million new cases are reported every year in India, with around 12% of death associated with people of age less than 40 years (Anand et al. 2001). Various key factors are ascribed as major causes that associated instigate strokes, such as alcohol consumption, higher intake of cholesterol, inherent factors, cardiovascular problems, diabetes, obesity, and depression (Must et al. 1999). Consequently, gender-based variances in the prevalence of stroke have been acknowledged, as strokes are more prominent in males in comparison to females. Despite this fact, mortality rates are way higher among females due to bleeding during pregnancy and some other similar complications (Palomeras Soler and Casado Ruiz 2010). The chances of stroke prevalence increase with age; but epidemiological variances occur with some populations, showing a greater risk of strokes at earlier ages also (Ovbiagele and Nguyen-Huynh 2011).

The occurrence of strokes is usually silent, asymptomatic, and its onset is unexpected. Patients may report weakness in their muscles, feel dizziness, blurred vision, and may experience difficulties in speaking. Also, a person may lose his ability to stay conscious (Lisabeth et al. 2009). Various imaging and diagnostic approaches have been reported in the literature to strengthen the fact that nanoparticles bolster and improve the diagnosis of stroke and its risk. However, it is said that mostly imaging techniques have been used to diagnose the reason for stroke instead of anticipation of the risk of stroke. The conventional trajectories have been used previously and reported to cause post-ischemic cerebral damage. Till date, the Food and Drug Administration (FDA)-approved therapy for ischemic stroke comprises of the usage of tissue plasminogen activator (tPA), a thrombolytic agent, also the use of mechanical thrombectomy (Anand et al. 2001; Mozaffarian et al. 2015). Even though blood flow can be regained by these methods and decrease the cell death in the "penumbra' (a region nearby the ischemic core), firstly, they are restricted to some side effects such as cerebral hemorrhage, and secondly, the reality is that not every patient can undergo any of these therapies. Most of the patients reach the physician after 3 h, which is inappropriate for tPA administration. Although it limited therapeutic application, there are high chances that cerebral hemorrhage may occur during the treatment. Nonetheless, the fact that blood may be restored, subsequently, may give rise to secondary damage due to the generation of reactive oxygen species and some inflammatory moderators in the ischemic region (Lin and Wang 2016).

Most successful approaches to cope up with reperfusion injury occur due to the elimination of clot which is now under vigorous investigation. However, the diagnosis after stroke and problems encountered in the management of post-stroke, combined with the barriers posed by the brain, and therefore, complete recovery of the patient remains poor. Thus, there is a crucial need to go for actual effective approaches to overcome the present lacuna in imaging, diagnosis, and treatment of cerebral ischemia. An emerging platform has been reported in which therapeutic drug is co-delivered with the diagnostic agent to a specific target site in various brain disorders just to monitor the real-time effectiveness of therapeutic molecule; however, the challenge is to find the perfect combination of therapeutic and imaging agent for synergistic effects as well to ensure the co-delivery of the therapeutic agent to the ischemic regions by overcoming the formidable blood-brain barrier. Thus, nanotechnology-based interventions in the diagnosis and treatment of cerebral ischemia are of paramount importance.

Nowadays, the use of advanced technologies is to determine the degree of impairment due to ischemia, and also to tailor personalized nanotherapeutics for patients. Currently, various imaging techniques such as computed tomography (CT), intravital microscopy (IVM), positron emission tomography (PET), and very recently developed magnetic particles imaging (MPI) are used, not only in diagnosis and imaging but also to anticipate the susceptibility/predisposition/propensity risk of occurrence of another stroke/stroke recurrence/stroke relapse. Hence, the use of nanostructured systems that can transport both therapeutic and imaging agents to the targeted site via the blood-brain barrier might be an exhilarating therapeutic

outlook for the cure of cerebral ischemia. Also, in-depth knowledge of cerebral ischemia pathophysiology and presently available systems are highly encouraged to plan/implement solutions to fill in the existing lacuna. The following sections illustrate a holistic overview of ischemic brain disorders, existing therapeutic and diagnostic trajectories, and the interventions of nanoformulations that had been developed till now.

3.2 Pathophysiology of Cerebral Ischemia

Major pathophysiological processes involved in cerebral ischemia include energy failure, disruption of cellular ion homeostasis, acidosis, increased concentration of intracellular calcium, excitotoxicity, and free radical-mediated toxicity. Stroke arises due to insufficient blood flow to the brain tissue leading to energy failure and is the primary event contributing to much of the pathophysiology of ischemia (Rhim et al. 2013). Increased Ca^{2+} ion as a result of reduced energy triggers the formation of free radicals which further increases Ca^{2+} concentration. Depleted energy level leads to loss of ion homeostasis, glutamate-mediated excitotoxicity, and increased concentration of Ca^{2+} which lead to even more excitotoxicity. Brain damage attributed by each of the processes mentioned above can be different depending upon the related etiology – hypoglycemia, hypoxia, and ischemia (Siesjo 1992a, b). The reduction in blood flow leads to lack of oxygen and glucose supply which are the basic requirements for the production of ATP (Siesjo 1992b; Tymianski and Tator 1996). No ATP production further leads to decreased protein synthesis, extra- and intracellular acidosis, initiation of anaerobic glycolysis leading to the formation of lactic acid and disbalance in ionic homeostasis. Ischemia increases membrane permeability and ATP shortage which disrupt ionic gradient and drive K^+ out and Na^+ and Cl^- in, contradictory to normal conditions, which further lead to depolarization of the membrane, trigger glutamate release, and increase Ca^{2+} concentration. The abundant glutamate receptors in the extracellular space get stimulated like NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors as a consequence of ionic imbalance. This results in the enhancement of calcium, sodium, and water influx into neurons. DNA and mitochondrial damage may occur due to nitric oxide (NO), free radicals formed due to the activation of several proteolytic enzymes such as lipases, nucleases, etc. (Moskowitz et al. 2010). The schematic illustration of the mechanism is shown in Fig. 3.1.

Neuronal swelling may occur due to K^+ and water retention. Inflammation is initiated within a few hours of the ischemic attack. Inflammatory markers such as cytokines (TNF-α, IL-1, IL-6, TGF-β), inducible neuronal nitric oxide synthase (iNOS) and eicosanoids, and cell adhesion molecules are produced by the cells present in the brain such as the endothelial cells, microglial cells, astrocytes, and leukocytes which accelerate the neuronal death due to the oxidative stress (Wang et al. 2007). Release of neurotransmitter is physiological signal transduction due to depolarization of synaptic membrane with increased/elevated Ca^{2+}. During

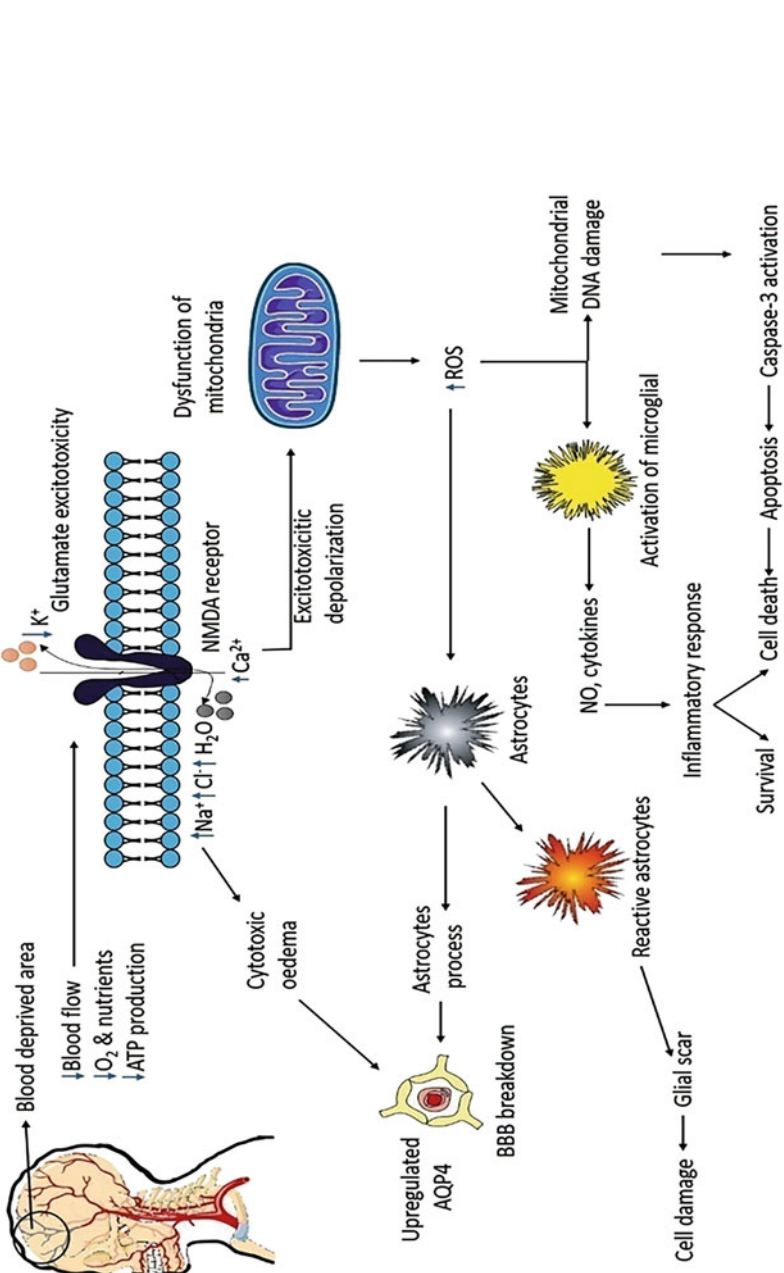

Fig. 3.1 Neuropathological mechanism of cerebral ischemia. Some key events can lead to cell death such as blood-deprived condition in the brain, consequently lack of oxygen supply leads to various physiological maladies and ionic homeostasis imbalance such as the increase in the concentration of calcium and sodium ions, and decrease in potassium ion concentration. Ultimately, the imbalance of the physiological process leads to mitochondrial damage, thereby, resulting in cell death

ischemia, an abnormal and probably prolonged depolarization of neurons, as well as the changes in Ca^{2+} homeostasis, causes the release of non-physiological amounts of many neurotransmitters including glutamate (Siesjo 1992a, b; Tymianski and Tator 1996; Macdonald and Stoodley 1998). The increased concentration also affects the release of dopamine, acetylcholine, and γ-aminobutyric acid (Globus et al. 1988; Benveniste et al. 1984). Synthesis of dopamine, acetylcholine, and other neurotransmitters, oxygen, and functioning of the Krebs cycle is likely to be decreased by ischemia as it requires ATP, thereby, ultimately hampering the normal physiology of the brain and eventually leading to the cerebral attack.

3.3 Conventional Therapeutic Trajectory

The main focus of the conventional therapeutic approach is to manage the cerebral stroke and related complications by the administration of a therapeutic agent to dissolve the clot in the brain to mitigate the neuro-inflammation by either intravenous (IV) thrombolysis or endovascular therapy. The important factor that regulates the efficacy of a given therapy is "fast dissolution of clot" which means it is ensured that clot should be dissolved as fast as possible within a narrow window of time just to ensure minimal damage to the brain (Wardlaw et al. 2013). Moreover, there are some other options also available to restore the blood flow in blood vessels such as stenting and surgical interventions (Gralla et al. 2012). However, it has been observed that in most of the cases these interventions had been proved as drastically failed strategies and could not prevent disability or the death of the patient.

Therefore, it is a shortcoming of the therapeutic approach that effective treatment along with its imaging and diagnosis is still absent from the picture. Although, after many successful years, a lot of advanced imaging and diagnosis strategies have developed now and proven to be effective in curbing the real-time and in-advance maladies associated with cerebral ischemia. Apart from this, in a conventional trajectory, the approach remains quite unsuccessful without the use of nanostructural carriers for the delivery of effective therapeutic agents and imaging agents (Tapeinos et al. 2020; Kaviarasi et al. 2019).

3.3.1 Therapeutic Agents for the Treatment of Cerebral Ischemia

This section covers a brief account of various therapeutic agents and their roles for the treatment of cerebral ischemia stroke and related complications.

3.3.1.1 Thrombolytic Drugs

Since the main cause of cerebral stroke is vascular clogging/occlusion due to clot formation/the formation of clot in blood vessels caused by the factors which are responsible for blood coagulation and fibrinolysis present in the biological system itself. Plasminogen cleaves and forms plasmin in the presence of plasminogen activator and this serves as the building block for the native process of fibrinolysis.

The degradation of solid fibrin into soluble products processed by the action of plasmin, and when there is a disruption in the balance between coagulation and fibrinolysis, lead to some serious complications including cardiac stroke and cerebral ischemia. Additionally, the majority of the ischemic stroke cases arise due to the occlusion of blood vessels resulting in a thrombus or thromboembolus. The two main plasminogen activators present in the biological system are tPA and urokinase that trigger the fibrinolysis cascade process. The usage of tPA has been extensively reported as a clot-busting agent by intravenous route or via endovascular technique (Cesarman-Maus and Hajjar 2005).

Over the years, numerous new thrombolytic agents have been established for both fibrin-specific and non-specific categories. Apart from the benefits of these agents, all these agents have been reported to have a high risk of stroke and intracerebral hemorrhages (Lansberg et al. 2007). Therefore, there is an urgent call to look for the targeted delivery of these agents with some properties such as immediate release and clot dissolution.

3.3.1.2 Neuroprotective Drugs

Neuroinflammation is a key player in supplementing the lethal effects of cerebral ischemia and stroke by encouraging irreversible impairment of neuronal functions through the production of reactive oxygen species and inflammation-induced cytokines (Poupot et al. 2018). These agents are classified based on their activity at the initial stage of protection. But these drugs are not meant to be used alone in stroke therapy; therefore, these agents are used in combination with other drugs such as free radical scavengers, calcium chelators, nitric oxide antagonists, and many more. Additionally, it has been reported that minocycline is a candidate which can be used for cerebral stroke therapy because of its ability to penetrate/breach/cross the blood-brain barrier and mitigate the activity of microglia, circumvent glutamate-mediated neurotoxicity, as well as block caspase-dependent apoptosis (Kikuchi et al. 2012). However, some clinical trials have not shown promising results by the use of the above-discussed class of drugs, since their risks outweighed the positive results, and therefore, these classes of drugs have not gained/garnered much interest from clinicians (Mayo Clinic n.d.).

3.3.1.3 Platelet Aggregation Blockers

Another strategy for the prevention of stroke could also be achieved by the use of antiplatelet drugs which inhibit the aggregation of platelets. One of the most extensively used anti-platelet drugs is aspirin. The main mechanism of this drug is to avoid clot formation by blocking the process of platelet adhesion and circumventing recurrent thrombosis (Kaviarasi et al. 2019). Moreover, the combination of two therapies such as clot dissolution therapy and anti-platelet drugs could improve the outcomes as compared to monotherapy; however, it may cause severe side-effects such as bleeding (Bath et al. 2018).

3.3.2 The Limitations Associated with the Conventional Trajectory

It has been evident from the research of various groups that the administration of a single drug is not much effective in the treatment of stroke and its related complications; however, a cocktail of two or more therapeutic agents having a different mechanism of action could be an effective approach (Knecht et al. 2018). The administration of these agents causes system toxicity due to the triggering of plasminogen in areas with normal blood circulation that ultimately leads to/culminates in hemorrhagic conditions. Therefore, the most preferred treatment of stroke is to deliver the therapeutic agent in combination with other therapeutic agents to the specific site to curb the stroke complications (Bansal et al. 2013).

Additionally, another main limitation in the treatment of stroke is to avoid the barriers offered by the brain, i.e., blood-brain barrier (BBB). Therefore, for effective therapy, it is must for the therapeutic agent to cross three barriers, first is the blood-brain interstitial fluid which is separated by BBB, second is the blood-cerebrospinal fluid barrier, and the last is an arachnoid epithelial layer. The structure of BBB is complex, composed of various types of cells such as endothelial cells, perivascular mast cells, some astroglial cells, and pericytes (Abbott et al. 2010). The tightness of this barrier is attributed to the presence of tight and adherens junctions of capillary endothelial cells. There are three important proteins available at BBB namely occludin, junctional adhesion molecules, and claudins (Redzic 2011). In regard to the permeation via BBB, some transport systems are actively involved in transporting the drug molecules through the tight junctions of BBB. However, the structure of these transport systems tightly maintained and regulated; therefore, many times the permeation of drug molecules across the BBB is difficult, thereby, there is an urgent requirement to look toward the advanced nano-therapeutics which are expected to cross the blood-brain barrier and could reach the target ischemic site for the improved therapy. Additionally, advanced nano-therapeutics along with the imaging and diagnosis techniques could lead to the successful and effective treatment of ischemic stroke (Prasad et al. 2018).

3.4 Nanotechnology – An Unfold Blueprint in Imaging and Treatment of Cerebral Ischemia

Nanodimensional vectors have the unique ability to encapsulate both hydrophilic and lipophilic drugs inside their core, thereby, increasing their circulation stability as well as their solubility problem that restricts the hydrophilic drugs to show their therapeutic effects. Furthermore, it is possible to encapsulate multiple therapeutic agents in a single core of nanocarrier to improve therapeutic efficiency and enable them to target stroke (Hu et al. 2012). Nevertheless, many factors can influence the internalization of these nanoparticles including a charge on the surface of nanoparticles, lipophilicity, and shape. Additionally, one of the important factors that affect the internalization of nanocarrier into the tight junctions and pericytes of the blood-brain barrier is the size of nanocarriers (Christiane et al. 2015). Moreover,

the surface of these nanocarriers can be customized to graft ligands for specific/cognate target receptor interactions that can permit site-specific binding of these nanocarriers to deliver therapeutic drugs for the treatment of cerebral ischemia (Zhao et al. 2016). Similarly, the modification of the surface of these nanocarriers and incorporation of therapeutic moiety with luminescent dye or contrasting agent which is compatible with the biological system could impart better and positive outcomes for the imaging and diagnosis of cerebral ischemia along with its treatment. Further, the advancement could be achieved in nanotechnology in terms of invention of better and superior nanovectors for the release of therapeutic moiety based on the response triggered by the cells at a specific site. Still, there is a lot of advancement in nanocarrier systems reported in the literature, out of which one of the instances is stimuli-responsive nanocarriers that can allow temporal control of therapeutic moiety release which could be of significant use in terms of stroke therapy (Lv et al. 2018a, b; Gulfam et al. 2019). An extensive repertoire of nanocarriers which are made up of natural and synthetic origin with different shapes, sizes, and modifications over surface have been investigated for the delivery of therapeutic moiety in a wide range of diseases (Banik et al. 2016). Taking into consideration cerebral stroke therapy, liposomal and polymeric nanoparticles are mostly employed systems for its treatment. The important role of nanocarriers to target the specific site and modification with particular ligands enable them to target the thrombus site which could be a plausible approach for the treatment of cerebral ischemic stroke (Han et al. 2016). The recent advancements of nanoparticles for imaging cerebral ischemia are tabulated in Table 3.1.

Albeit, the important multipurpose usage of these nanovectors has been stated by many researchers across the literature; however, it should not be overlooked that one of the major promising features of these nanovectors is multimodality. The incorporation of both therapeutic agents and diagnostic agents in the same nanovector is a robust technique to improve the therapeutic as well as diagnostic efficacy against many lethal diseases (Bonnard et al. 2019). Therefore, the function of these nanovectors to enhance retention time in circulation, bioavailability, also would endow additional aid for the simultaneous multiple drug delivery and *in vivo* monitoring or diagnosis of cerebral stroke. Furthermore, these nanovectors occasionally provide an additional advantage as bestowed upon by their compositions. For this case, Liu et al. presented a unique strategy for the imaging of damage in BBB with the use of superparamagnetic iron oxide nanoparticles (SPIONs) which were used as contrast agents. The use of SPIONs as imaging agents made it possible to monitor the modifications in BBB and ischemic lesions with MRI; additionally, the monitoring could last for 24 h after the single administration of PEGylated SPIONs in an animal model (Liu et al. 2014). Thus, such kind of monitoring of alteration in BBB permeability could be a plausible way to investigate the problem and could be used to begin a perfect therapeutic regimen after the diagnosis. Similarly, Kim et al. developed a computed tomography-based technique to directly monitor cerebrovascular thrombi and assist in thrombolytic therapy. In this study, they synthesized chitosan-coated gold nanoparticles for the direct imaging of thrombosis in the animal model. The following sections summarize numerous nanoparticles used in the therapy of cerebral stroke (Kim et al. 2015).

Table 3.1 List of recent advancements of nanoparticles for imaging of cerebral ischemia

S. No.	Nanoparticles	Imaging technique	Salient features	References
1.	Iron oxide nanoparticles	MRI	5-fold increase in total glioma exposure Improvement in target selectivity index by 3.6-fold	Conte et al. (2019)
2.	Superparamagnetic iron oxide nanoparticles	Microwave imaging	Signal was enhanced after the administration of these nanoparticles	Hudson et al. (2019)
3.	Ultra-small magnetic dual contrast iron oxide nanoparticles	MRI	Improved targeting ability in *in vivo* Improved diagnostic accuracy	Ta et al. (2017)
4.	Superparamagnetic iron oxide nanoparticles loaded micelles	MRI	Fluorescent Nile dye & SPIONs loaded simultaneously in micelles to label the neural stem cells (NSCs) Delivery to NSCs successfully tracked by SPIONs loaded micelles	Lu et al. (2017)
5.	Fluorescein hyaluronic acid gold nanoparticles	Fluorescein imaging	Exhibited strong fluorescein signal via cleavage of gold NPs conjugation and hyaluronic acid Investigated the damage caused by ROS in the brain up to 41 h	Lee et al. (2009)
6.	Thrombin-activatable fluorescent peptide encapsulated silica-coated gold nanoparticles	Near-infrared fluorescence/ micro-computed tomography	Showed ~ 30-fold higher near-infrared fluorescence intensity Successfully accumulated in thrombus and helped in differentiating the thrombotic lesion from other tissues	Kwon et al. (2018)
7.	Functionalized gold nanoparticles	MRI	Enhanced T_1 relaxivity and outstanding cellular uptake Almost 70% of cells were identified correctly by these NPs and proved as an effective and reliable technique	Nicholls et al. (2016)

(continued)

Table 3.1 (continued)

S. No.	Nanoparticles	Imaging technique	Salient features	References
8.	PEGylated $BaHoF_5$ NPs	CT angiography/ CT-perfusion imaging	Improved diagnostic sensitivity and accuracy towards cerebral ischemia Low dose required as compared to conventional iodinated contrasting agents	Wang et al. (2015)
9.	Glycol-chitosan-coated gold NPs	CT imaging	Fibrin binding capacity was higher for Fib-GC-AuNPs than GC-AuNPs Fib-GC-AuNPs endow prompt detection of cerebral thrombi	Kim et al. (2015)
10.	Glucose-coated gold NPs (GNPs)	CT imaging	Coating of exosomes using GNPs was performed GNPs administered via intranasal route showed higher accumulation in the brain as compared to intravenous administration Increased accumulation at the ischemic region as compared to non-specific sites	Betzer et al. (2017)
11.	Gold nanocoral	Surface-enhanced Raman spectroscopy	Deposition of gold on self-assembled boehmite nanostructures Enabled improved visualization of cerebral ischemia	Yamazoe et al. (2014)
12.	DCDPP-2TPA NPs	3-Photon fluorescence microscopic imaging	Superior visualization was achieved into deeper tissue Even deeper cerebral vasculature and blood vessels of 2.4 μm were clearly visible	Wang et al. (2017)
13.	DMCA-MnO-Fe_3O_4	MRI	No local damage by MR radiations Excellent T_1 and T_2 imaging at higher resolution of ependymal brain cells.	Peng et al. (2017)

3.4.1 Polymeric Nanoparticles

Polymeric nanoparticles are amazing nanovectors in which drugs can be loaded by either way as encapsulation or covalent bonding and could be modified to respond to

stimuli for the sustained and controlled release of loaded drug (Gao et al. 2018). The release of various drugs is depended on the polymer involved in the synthesis of such nanoparticles. There have been a plethora of polymers reported in the literature which could be used to synthesize the nanoparticles, to enhance the kinetics of drug and endow a successful therapy for the targeted disease (Bennet and Kim 2014). In the context of cerebral ischemia, delivery of therapeutic moiety via nanoparticles is highly encouraged just because of the tendency of these nanoparticles to be retained in systemic circulation and cross the blood-brain barrier to reach the target site for improved therapeutic effects. Out of many polymers, one of the commonly used polymers is poly(lactic-co-glycolic acid) (PLGA) which is an FDA approved biodegradable polymer. The lysis of PLGA nanoparticle results in a reduction in molecular weight and generation of space between the polymer chains, thereby, facilitating the release of therapeutic moiety from the created voids. The amount of lactide in the copolymer affects the degradation process of PLGA, as a higher amount of lactide decreases the hydrolysis due to its hydrophobic nature (Ghitman et al. 2020). Furthermore, the nature of the polymer, as well as its by-product of hydrolysis, does not endow any kind of toxic effects in the systemic circulation or target site, therefore, has been the best choice of researcher for the drug delivery purpose (Wang et al. 2010).

Taking into consideration, the role of PLGA nanoparticles in stroke has been widely discussed in the literature. PLGA nanoparticles have been used to deliver the antioxidants and neuroprotectants to alleviate oxidative stress-facilitated neural damage and mitigate the neural-inflammation/neuroinflammation. For example, Reddy et al. developed antioxidant enzyme encapsulated PLGA nanoparticles to scavenge the free radicals and was investigated in a rat focal cerebral ischemia-reperfusion injury model. The nanoparticles were administered by intra-carotid artery during the reperfusion procedure. The findings of this study revealed that enzyme encapsulated in PLGA nanoparticles showed greater efficiency in decreasing the size of infarct when it was compared with the free enzyme (Reddy and Labhasetwar 2009). Additionally, the survival of animals was relatively higher in the nanoparticle-treated animals as compared to another group. Even though the delivery of these nanoparticles by the intra-carotid route helps to maintain the integrity of the blood-brain barrier, thereby, it reduces the chances of edema formation prevalence (Reddy and Labhasetwar 2009).

Similarly, Petro et al. developed antioxidant enzyme encapsulated loaded biodegradable PLGA nanoparticles for the management of thromboembolic stroke. These nanoparticles were administered in animals after 3h injection of tissue plasminogen activator. The histopathological studies of the brain revealed higher glial fibrillary acidic protein-positive cells when it was compared with animals without treatment or treated with tPA only. The higher number of SOX-2 and Nestin positive cells were mobilized from subventricular region to rostral migratory stream reported after the co-administration of tPA and enzyme-loaded nanoparticles as compared to the alone nanoparticles-treated group and tPA-treated group. Also, the treatment with the combination of nanoparticles and tPA showed a reduction in cells which have caspase and neutrophils as compared with other groups and ultimately inhibited

the swelling of the hippocampus (Petro et al. 2016). Enzyme encapsulated polymeric nanoparticles experience a problem as enzymes are prone to slight variation in the biological environment. Thus, enzyme mimicking has been explored to circumvent these issues of enzymes. Fabian et al. in their novel attempt reported that they designed a hydrophilic cluster which was formed by acid treatment of nanotubes. These nanotubes were used as SOD enzyme mimics and delivered to the site of infarction by the use of PLGA nanoparticles in cerebral artery occlusion induced in streptozotocin-treated rodent models. The findings of this study revealed that these nanoparticles exhibited higher efficacy in the reduction of infarct size and mitigation of inflammation disclosing their effective potential for post-stroke therapy (Fabian et al. 2018).

Whereas, another study of PLGA nanoparticles with the encapsulation of two different fractions of polyphenolic compound "curcumin" which disclosed that encapsulation of curcumin prevents the hydrogen peroxide facilitated phosphorylation of Akt/tau proteins in SK-N-SH cells (Ahmad et al. 2016). In another study, the efficacy of melatonin-encapsulated PLGA nanoparticles against cerebral stroke was evaluated. Even though melatonin suffers from a readily degradation problem when it is exposed to sunlight and short-half life, the encapsulation of this molecule in nanoparticles showed promising results in female rat models with cerebral ischemia-reperfusion injury. The findings of this research disclosed that the stability was improved and normal levels of anti-oxidants were also restored except few such as lipid peroxidation and mitochondrial dysfunction (Sarkar et al. 2017).

Similarly, a phytoconstituent "safranal" was investigated for its antioxidant property and was proved effective in cerebral stroke induced in rats. In this study, the nanoemulsion was prepared using a blend of surfactant and co-surfactant namely Tween 20, Cremophor EL, and Labrasol (Ahmad et al. 2017). In another study, eugenol was used to investigate its potential in Wistar rat brain and outcomes showed that the bioavailability of eugenol was improved in the rat brain when it was encapsulated in polymeric nanoparticles (Ahmad et al. 2018). Recently, the investigation of phytochemicals has drastically increased in therapeutic applications. On this basis, a phytochemical "tanshinone IIA", an active constituent available in the *Salvia miltiorrhiza* plant exhibits anti-inflammatory effects via the suppression of enzyme nitric oxide synthase and phosphorylation of VEFG and NFκB (Zhang et al. 2018). Although this molecule poses a permeation problem across BBB and exhibits reduced circulation time in the systemic circulation, therefore, to improve its permeation via BBB, it was conjugated with polyethylene glycol (PEG) and coupled with albumin nanoparticles for the effective delivery of this molecule across BBB to infarct site, thus, providing better therapeutic effects against ischemia. Hence, polymeric nanoparticles are the perfect/ideal candidates for the encapsulation of therapeutic cargoes/moiety to target cerebral ischemia (Sarmah et al. 2017).

3.4.2 Lipid-based Nanoparticles

Lipid-based nanoparticles have gained a lot of attention from scientists due to their unique structure and their composition, comprised of mostly natural or synthetic lipids. Some carriers covered in lipid-based nanoparticles category are liposomes, solid lipid nanoparticles, and nanostructured lipid carriers (Puri et al. 2009). The unique ability to carry both hydrophilic and hydrophobic drugs across the membrane with no toxicity enables them to be a perfect choice for drug delivery across the blood-brain barrier (Kumar 2019). Additionally, lipid-based nanoparticles are flexible to chemical modifications to evade/escape/circumvent the recognition by the immune system (Mizrahy et al. 2017). In the context of cerebral ischemia, lipid-based nanocarriers are effective carriers just because of their lipidic nature and due to this, it can permeate through the blood-brain barrier and can carry the therapeutic agent to the target site/site of action, thereby increasing the bioavailability with more/augmented therapeutic outcomes (Sadegh Malvajerd et al. 2019). There have been numerous lipid-based nanocarriers investigated for the management of cerebral stroke and associated complications. Partoazar et al. prepared cyclosporine-loaded liposomes for post-stroke complications. This liposomal formulation was synthesized by the blend of different lipids such as phosphatidylserine (PS), cholesterol, and distearoylphosphoethanolamine-PEG-2000 (DSPE-PEG-200). When it was administered at a dose of 2.5 mg/kg to animals, occlusion of the middle cerebral artery occurred for 90 mins followed by the reperfusion for 48 mins to mediate reperfusion injury just as post-stroke conditions. The group which was treated with liposomal formulation exhibited significantly better outcomes in terms of size of infarct and brain edema (Partoazar et al. 2017). Li et al. encapsulated a polyphenolic compound baicalin that has anti-oxidant potential for the management of cerebral ischemia. The therapeutic efficacy of baicalin was restricted due to its poor solubility and the encapsulation in liposomes circumvent this problem, therefore it was successfully encapsulated in lecithin and cholesterol liposomes. The findings of this study revealed that when liposomes were administered in animals having an ischemia-reperfusion injury, a significant improvement was observed in the retention of baicalin in the brain tissues as compared to free drug agents (Li et al. 2018). In another study, Adibhatla et al. investigated the outcomes by encapsulation of a neuroprotective agent “citicoline” in liposomes. The preparation of liposomes was done by using dipalmitoylphosphatidylcholine (DPPC), cholesterol, and ganglioside which were later investigated in the focal cerebral ischemia animal model. The investigation revealed that citicoline-loaded liposomes reduced the infarct area by almost 2.5 folds in comparison to free citicoline (Li et al. 2016). Neuroinflammation is a common malady that comes up in the brain which worsens/aggravates/exacerbates neurodegeneration (Ransohoff 2016). To reverse the pro-inflammatory problems, siRNA was encapsulated in nanoemulsion which was designed using phospholipid and Tween 80 surfactants to mitigate neuroinflammation. The findings disclosed the higher internalization of siRNA nanoemulsion as compared to free siRNA, resulting in decreased inflammatory conditions. Additionally, the administration of siRNA encapsulated nanoemulsion significantly silenced the expression of

TNF-α (Yadav et al. 2016). The surface modification and composition of lipids could alter the release of drug, pharmacokinetics, and biodistribution of therapeutic agents among different tissues. For instance, differently charged liposomes were employed for the encapsulation of simvastatin. Therefore, findings revealed that liposomes owning neutral and negative charges showed better accumulation in the brain after IV administration in the animal model. Furthermore, the same investigation disclosed that neutral liposomes showed better bioavailability as compared to charged liposomes (Campos-Martorell et al. 2016). The key limitation of the liposomal system is its poor circulation in the biological system. However, the incorporation of such systems with PEG-linked lipids has been explored to improve their circulation time and lifetime by delaying the opsonization (Yan et al. 2005).

Palazzo et al. developed injectable liposome and drug-cyclodextrin-in-liposome (DCL) formulations to improve the estetrol permeation through the blood-brain barrier. The investigation/testing of these formulations was performed against human blood-brain cell line/human cerebral microvascular endothelial cell line (hCMEC/D3). The investigation disclosed the particle size, polydispersity index (PDI), and zeta potential which were 150 nm, 0.10, and +31 mV, respectively for all formulations. The findings revealed no toxicity against the cell lines and showed no platelet aggregation and hemolysis. Hence, these formulations proved as an effective approach to deliver estrogen to the brain by overcoming the blood-brain barrier (Palazzo et al. 2019). Ishii et al. investigated the role of PEGylated liposomes in the treatment of cerebral ischemia-reperfusion injury. Additionally, the study was aimed to disrupt the blood-brain barrier to enhance the therapeutic effects of the neuroprotectant which suffers from permeation problems across BBB. The findings disclosed that reperfusion began in transient middle cerebral artery occlusion (t-MCAO) after the administration of PEGylated liposomes at different time points in the animal model. The liposomes showed higher accumulation in the ischemic hemisphere at the initial stage and were retained for 24 h in the lesion after injection. Furthermore, this group investigated the potential of asialo-erythropoietin (AEPO) PEGylated liposomes for the treatment of cerebral ischemia-reperfusion injury. Later, the results disclosed that AEPO-liposomal formulation can successfully treat cerebral ischemia-reperfusion injury (Ishii et al. 2012).

Kakkar et al. performed the investigation of curcumin-loaded lipid-based nanoparticles against cerebral ischemia in the rat model. The preparation of curcumin-encapsulated solid lipid nanoparticles (SLNs) comprises of solid lipid (0.58%) and surfactant (45.45%). The effects of these drug-loaded SLNs were evaluated in terms of behavioral change and elevated levels of various enzymes. The findings revealed that these drug-loaded SLNs improved 90% cognition and inhibited the cerebral acetylcholinesterase enzyme activity by 52% against implicated in cerebral ischemia-reperfusion injury. Also, the improvement in neurological score was reported by 79%. Furthermore, results of gamma-scintigraphy revealed the improvement in brain bioavailability by 16.4-fold and 30-fold after the administration of curcumin-SLNs orally and intravenously, respectively. Hence, the findings divulged the suitability of lipid-based nanoparticles for the delivery of curcumin to improve various parameters to treat cerebral ischemia-reperfusion injury

(Kakkar et al. 2013). Wu et al. developed nanostructured lipid carriers (NLCs) for the delivery of salvianolic acid B (Sal B) and baicalin (BA) to the brain for the management of neuronal damage and ameliorate cerebral ischemia-reperfusion injury. These nanoparticles modified with transferrin receptor monoclonal antibody OX26 (OX26-BA/Sal B-NLCs) achieved the targeted delivery. This study was evaluated in terms of *in vitro*/*in vivo* targeting ability, pharmacodynamics and pharmacokinetics and the release of these nanoparticles followed the Weibull model. Some findings revealed that NLCs modified with OX26 exhibited a significant increase in the concentration of BA in brain tissue as compared to the unmodified group. Hence, the discussed findings disclosed that these modified NLCs loaded with two drugs could improve the delivery of these drugs to the brain by overcoming the various pathways (Wu et al. 2019).

Yuan et al. developed PEG-modified and tanshinol borneol l ester (DBZ)-loaded nanostructured lipid carriers (DBZ-PEG-NLCs) and DBZ-NLCs to investigate their role in C57BL/6 mouse model of cerebral ischemia. The characterization of these lipid carriers was performed in terms of particle size, zeta potential, entrapment efficiency, and drug loading. The findings disclosed no significant difference in the mean particle size and entrapment efficiency of DBZ-PEG-NLCs and DBZ-NLCs. The release was biphasic with an initial burst and followed by the sustained drug release. Additionally, higher concentration and retention of DBZ were achieved in the brain and plasma upon the administration of DBZ-PEG-NLCs as compared to DBZ-NLCs and DBZ only. At last, these lipid-based nanoparticles divulged/showed an improvement in the antioxidant property as compared to DBZ and DBZ-NLCs in the animal model with ischemia injury by reducing the brain malondialdehyde and by improving the concentration of superoxide dismutase in the brain. Therefore, these NLCs could be employed as an effective carrier to deliver DBZ for the successful therapy of cerebral ischemia (Yuan et al. 2018).

3.4.3 Other Types of Nanoparticles

Apart from the polymeric and lipid-based nanoparticles, an extensive literature on other types of nanoparticles has been reported in the management of cerebral ischemia. Silica-based ceramic nanoparticles have also been explored for the delivery of therapeutic agents to treat cerebral ischemia and associated complications. The unique structure of these nanoparticles which is a highly oriented porous network along with tuned pore dimension enables them to incorporate various distinct properties that ultimately helps in the delivery of drug (Torchilin 2006). Additionally, another group investigated silica-coated magnetic NPs coupled with tissue plasminogen activator (tPA) for the accumulation at thrombus site with the magnetic guidance. Later, the accumulation was established by employing microCT imaging (Chen et al. 2012). Also, carbon-based nanoparticles are another class of nanocarriers which could be investigated for cerebral ischemia and related complications.

A research group had investigated the nanotubes in cerebral ischemia in which vertically aligned carboxyl functionalized nanotubes were tuned with PEG chains and could be used as a successful carrier to deliver the glucocorticoid dexamethasone for an extended period of time (Tang et al. 2012). Another category of nanocarriers which can be used as a potential carrier to deliver the therapeutic agent in cerebral ischemia condition is exosomes. These are vesicular nanocarriers which are secreted by various cells. An investigation of exosomes was carried out for targeted delivery in the ischemic brain in which the exosomes were modified with rabies virus glycoprotein (RVG)-Lamp2b and loaded with microRNA miR-124 (Yang et al. 2017). Similarly, exosomes were coated with cyclic RGD peptide to target the ischemic region by the delivery of anti-inflammatory agent "curcumin" in the middle cerebral artery of mice (Tian et al. 2018). Also, another nanocarrier that responds/acts according to the higher level of thrombin and matrix metalloproteinase (MMP9) just to deliver the glyburide has been stated in the literature. The findings revealed that peptide that helps in targeting was reported to improve the permeability of nanocarriers through the blood-brain barrier with the help of CXCR4 receptor present in BBB (Guo et al. 2018). Virus-based nanoparticles have been explored extensively for the delivery of therapeutic agents. A research group investigated the virus-based nanoparticles incorporated with a streptokinase-a thrombolytic agent. These nanoparticles exhibited higher accumulation at the site of thrombus, thus ensuing better thrombolysis (Pitek et al. 2017). Mukesh et al. developed vitamin D_3 nanoemulsion for the treatment of cerebral ischemia. The characterization revealed that the mean particle size and zeta potential of the nanoformulation was around 50 nm and 13.77 mV respectively. The imaging of the therapy was achieved by using gamma scintigraphy, radiometry assay, and magnetic resonance imaging which revealed better disposition and accumulation of 99mTc-Vitamin D_3-nanoemulsion in brain tissue through the nasal route as compared to IV administration of 99mTc-Vitamin D_3 solution (Kumar et al. 2020).

Zhang et al. have developed an effective approach of nanocarriers-exosomes for the delivery of miR-210 to the ischemic brain by intravenous injection that subsequently induces focal angiogenesis. The findings of this study have revealed that the modification of exosomes with miR-210 can overcome the barrier problem imparted by blood-brain barrier and mitigate the symptoms of stroke by circumventing miRNA degradation (Zhang et al. 2019). Betzer et al. developed a strategy for non-invasive *in vivo* diagnosis, neuroimaging, and chasing of exosomes by use of glucose-coated gold nanoparticles (GNPs) labeling and CT imaging. The findings revealed that glucose-coated nanoparticles were uptaken into exosomes through an active and energy-dependent mechanism which is regulated by the glucose transporter and the enhancement in the labeling of exosomes by 5 nm GNPs resulted in improved accumulation in the brain and increased/conspicuous *in vivo* neuroimaging. Therefore, it was proved that exosome labeling strategy serves as powerful imaging, diagnosis, and treatment for various brain-related diseases (Betzer et al. 2017). Therefore, such types of nanocarrier could be used as a promising approach to diagnose and treat cerebral ischemia and related complications. The key hindrance while the delivery of the drug to the brain could be overcome by using such types of

nanocarrier which would ultimately improve the therapeutic efficacy of the drug in cerebral ischemia.

3.5 Exploitation of Certain Advanced Technology for Imaging and Diagnosis of Cerebral Ischemia

The employment of nanotechnology for imaging and diagnosis emerges as an advanced field for effective management of cerebral ischemia. Along with nanoparticles carrying the drug, some imaging contrast agents can be modified and delivered to the systemic circulation for better imaging, diagnosis, and treatment of cerebral ischemia. Every developed imaging contrast agent is wisely fabricated for better efficacy at the minimum concentration of the imaging agent. Also, the functionalization can be achieved by using different advanced strategies such as by modification of nanocarriers that ultimately help in enhancing the drug and diagnostic agent circulation time in the blood and endow them an ability to permeate across the blood-brain barrier. Additionally, it provides them better contrast potential and enables them to be perfect to evaluate the ischemic damage and effect of nanotherapeutics. Many examples of smart nanotherapeutics and diagnostics have been reported. Therefore, this section of the chapter summarizes the recently developed advanced techniques for the diagnosis and therapy of cerebral ischemia. The schematic illustration of advanced nanotheranostics for the treatment of cerebral ischemia is shown in Fig. 3.2.

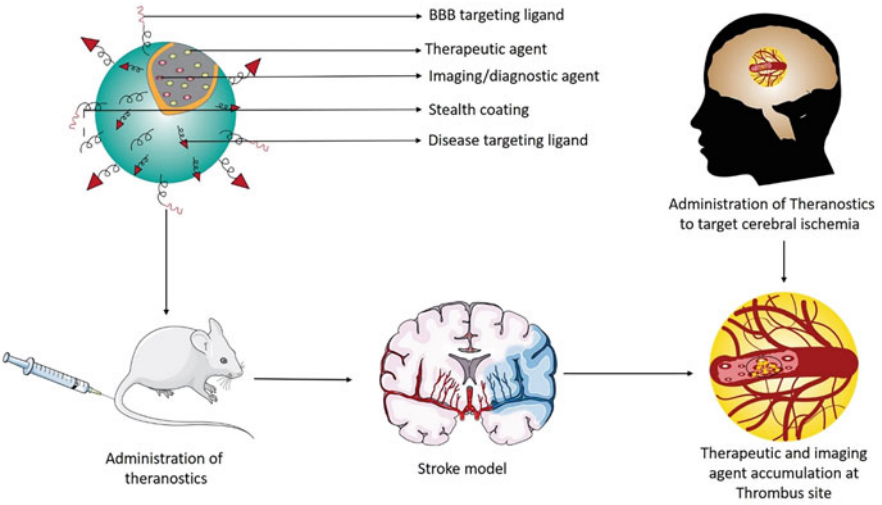

Fig. 3.2 Schematic illustration of advanced nanotheranostics for the treatment of cerebral ischemia. The dual loading of therapeutic and imaging agents endows dual targeting by accumulating at ischemic region/thrombus for the monitoring of outcomes of smart nanotheranostics and exerting therapeutic effects

3.5.1 Imaging/Diagnosis by Magnetic Resonance Imaging Technique

Magnetic resonance imaging (MRI) is an advanced non-invasive imaging technology which uses high-resolution 3D monitoring of various physiological and anatomical regions without exposing to damaging radiations. Additionally, this section also provides an overview on MRI method and its potential to diagnose and treat various complications related to cerebral ischemia, and the encapsulation of therapeutic agent in nanoparticles along with the contrasting agent as theranostics strategy. Finally, some light will be thrown on the recently developed nanotheranostics using MRI for cerebral ischemia.

MRI technology exploits a powerful magnetic field to trigger specific deviation in direction of the protons of H_2O that exist in the human body. More precisely, the patient is exposed to radio-frequency radiation, and some energy released as protons which line up with the magnetic field is efficiently detected by MRI sensors. Therefore, the extent of energy released, also the time needed by the protons to line up with the magnetic field, provides important information about the nature of molecules, enabling to differentiate various tissues such as differentiation of grey and white matter of the brain, etc. (Wang et al. 2016; Rudkin et al. 2018).

Relating to ischemic stroke, MRI is a robust and advanced imaging technique that allows the monitoring and detection of the shape and size of the infarcted region, perfused tissues, and the availability of thrombus. The estimation of size provides an important proof while defining the risks of hemorrhage as it primarily occurs in patients having larger infarcted areas (Lansberg et al. 2007). Furthermore, MRI enables to monitor the images of ischemic lesions, aberrations in chronic ischemia, and microbleeds, thereby highlighting significant information regarding the stroke mechanism and its plausible etiology. Thus, the presence of microbleeds is contemplated as an indicator of hemorrhage-vulnerable small vessel disease and is related with an enhanced frequency of cerebral hemorrhage. In comparison to computed tomography, MRI shows higher robustness and sensitivity toward the detection and monitoring the small ischemic lesions, also, it assists in distinguishing the small ischemic lesions and chronic lesions, even when they are restricted to brainstem and cerebellum (Vert et al. 2017). Moreover, it is also possible to characterize the lesions by lower sensitive techniques such as computed tomography as it is also a widespread, robust, sensitive, and easily available technique in emergency situations. There are certain benefits of CT over MRI such as faster acquisition time which is around 1–2 min for conventional CT, whereas when it is combined with non-enhanced CT, angiography, and CT perfusion its acquisition time increases to around 10 min. While considering this, it is important to mention and indicate a few particular centers which are recently developed MRI protocols, and lessening the imaging time to around 6 min.

Moreover, the advancement in techniques are able to determine the dynamic process happening during various phases of the cerebral ischemic stroke which are decisive for improving traditional therapies. Recent advancements in the field of nanotechnology have bolstered the progress of research in this arena. Researchers

have to be thankful for the recent advancement of nanotechnology in the biomedical field, as various different MRI diagnostic nanoplatform can readily target specific cell types and phenomena during cerebral ischemia and related complications. While contemplating this, SPIONs exhibit an important multimodal theranostics nanoplatform, which could be monitored in real-time imaging with the help of magnetic particles for the characterization of the level of disease occurrence and its etiology (Ludewig et al. 2017). Additionally, these nanoparticles are highly biocompatible, particularly when compared with other inorganic nanoparticles, and already got approval from FDA for the treatment of various diseases such as anemia and kidney-associated problems (Bobo et al. 2016; Bashir et al. 2015). Also, its effective diagnostic approach has been exploited for the real-time imaging of cerebral collaterals induced during ischemia after the occlusion of middle cerebral artery in animal models (Zhang et al. 2020). Specially, some collaterals are small in size around 50–400 nm which are developed during the acute phase and exhibit an important role in enabling perfusion of contiguous areas; however, it is very challenging to monitor these collaterals by MRI and CT due to their small size (Wang et al. 2018a, b).

Particularly, polyethylene glycol (PEG)-coated iron oxide magnetic nanoparticles having diameter around 6.4 nm were functionalized with peptide sequence for the purpose of targeting with the help of click reaction. The findings revealed that these developed nanoprobes exhibited successful targeting specificity and enabled them to monitor and image collateral dynamics after reperfusion. Apart from this, there have been various SPION formulations reported throughout the literature in which particular site has been targeted for superior imaging and delivering the contrasting and therapeutic agent in a single core of nanocarriers (Wang et al. 2018b; Mekawy et al. 2019; Gauberti et al. 2018; Duan et al. 2017; Feng et al. 2017; Farr et al. 2014). In concluding this, we are much confident that more SPIONs will come up in the biomedical field to diagnose and treat cerebral ischemia and related complications such as collaterals, inflammation, and apoptosis.

3.5.2 Imaging/Diagnosis by Computed Tomography

Computed tomography has been established as a gold standard technique in early-stage patients of cerebral ischemia (Kidwell et al. 2004). There are many organs and tissues which can be monitored and diagnosed without the involvement of contrasting agents (CAs). Monitoring and diagnosis of certain soft tissues and biological structures require the involvement of contrasting agents (Lusic and Grinstaff 2013). Therefore, the most common class of contrasting agents used in the imaging and diagnosis are iodinated compounds. Non-contrast-based CT is employed to measure the early sign of cerebral ischemia by determining parameters such as insular ribbon shape and hyperdense artery sign. There are lots of limitations of CT, such as higher doses are required due to low X-ray attenuation, toxicity may be induced due to high viscosity and osmolarity, and rapid clearance from the body due to its small size (Lusic and Grinstaff 2013). Additionally, the use of CAs for

CT/SPECT is even more complex due to the involvement of radioisotope-based contrasting agents. The involvement of these two could lead to an increased toxicological outcome resulting from undesired contact between two different CAs (Bonta and Wahl 2010). Thus, CAs should have the following properties such as high biocompatibility, better retention in organs, and optimum diagnosis and imaging properties. Out of many, glycol-chitosan-coated gold nanoparticles could be used as nano CAs in CT diagnosis and imaging. Specifically, gold nanoparticles functionalized by fibrinogen exhibited the ability to target cerebral thrombus as a diagnostic agent in cerebral ischemia (Kim et al. 2015).

Various liposomal-based contrasting agents in CT-based diagnostic and imaging have been reported throughout the literature, and investigated by researchers for a long time. These types of CAs involve the encapsulation of iodinated water-soluble compounds in the core of liposomes (Desser et al. 1999; Seltzer et al. 1984). In this context, one investigation reported the average size of liposomes around 140 nm which was employed for imaging and mapping the whole brain vasculature. Additionally, these carriers as contrasting agents were able to image and diagnose the small vessels up to 40 nm. Furthermore, it has been reported that liposomal-based imaging agents were able to monitor the aberrations that occurred in the brain vasculature of actin alpha-2 (ACTA2) mutant mice (Starosolski et al. 2015). In another study, PEG-coated gold (Au) nanoparticles have been investigated as CAs for improved CT imaging in animals. The findings of this study revealed that these AuNPs exhibited better X-ray attenuation ability due to their higher surface area as compared to bare Au nanoparticles. Additionally, these PEG-coated Au nanoparticles showed better CAs compatibilities in animal models, improved stability, and superior renal clearance as compared to bare AuNPs (Tang et al. 2017).

Therefore, CT-based imaging and diagnosis offer extensive information regarding the vasculature of the brain and any aberrations in the brain which result in various diseases including cerebral ischemia stroke. As the function of CT-based imaging differs as the region of imaging changes such as non-contrast CT is employed for the imaging in parenchymal tissues, having some advantages such as fast acquisition time and being highly sensitive to hemorrhages. For monitoring and imaging of the vasculature, CT angiography is performed which provides profound information related to aberrations in vasculature. There are some advantages of this technique such as fast acquisition time along with the quantification of vascular disease burden, i.e., the size of clot. CT perfusion is employed to image tissue perfusion.

3.5.3 Imaging/Diagnosis by Positron Emission Tomography

Positron emission tomography (PET) is one of the successful imaging techniques that can be used to image the brain penumbra. The basic working principle of PET is to detect the radioactive element known as radiotracers such as ^{18}F fluorodeoxyglucose, which provides a detailed evidence of biochemical processes at molecular and cellular levels. In comparison with other imaging techniques, PET

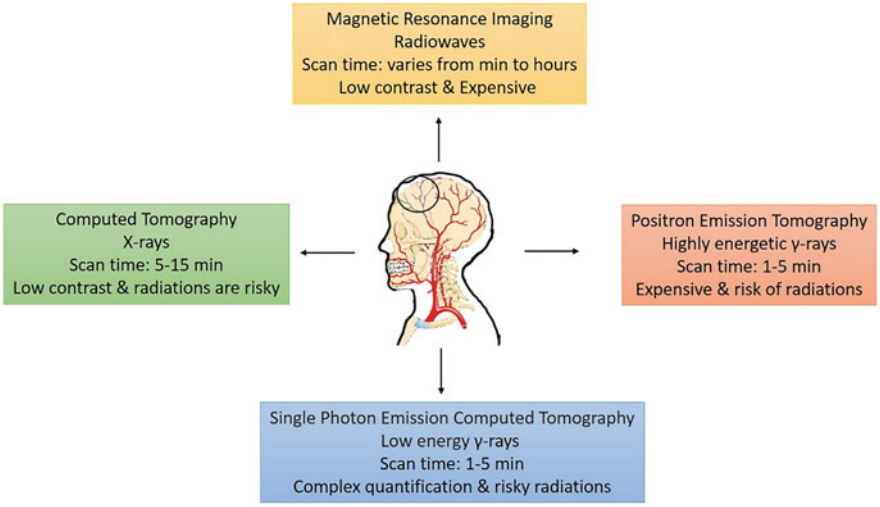

Fig. 3.3 Various imaging modalities for cerebral ischemia

is not only tracing the concentration of radiotracer administered in the body tissues, but it also detects the metabolic activity of tissues because the uptake of tracer is linked with glucose uptake. Even though PET is a robust and fast technique which delineates quantitative information, it has two main limitations that restrict its application in the biomedical field which are high cost and the emission of the radiations during application. Similarly, as other imaging techniques PET could also be used in combination with MRI or CT. Having high sensitivity and advancement, this technique can be employed to investigate various brain disorders such as Parkinson's, Alzheimer's, epilepsy, and others. The graphical representation of various imaging modalities is shown in Fig. 3.3.

In the context of ischemic stroke, PET can be employed to investigate the infarct volume and other complications associated with ischemia or reperfusion cascade. PET has been employed to explore the plausible role of receptors at the cellular level in brain-associated disorder (Moraga et al. 2015). It is also reported that the combination of these radiotracers enabled the investigation of proliferation and inflammation via translocation protein (TSPO) expression in toll-like receptor-positive mice. Along with the PET, MRI is also used to determine the extent of infarction in the brain. There are certain types of tracers which help in tracing the ischemic tissue; however, some tracers such as [^{11}C]PK11195 have better TSPO binding affinity, also it help in tracing glioma (Fujinaga et al. 2017).

Atherosclerotic plaque needs to be taken into consideration during the ischemic stroke, which might form just before and after the cerebral ischemic stroke(Vesey et al. 2017; Kelly et al. 2019). It has been reported that the formation of atherosclerotic plaque in the artery leads to inflammation which can be detected and diagnosed by an ^{18}F-FDG tracing agent (Vesey et al. 2017). Thus, the uptake of this tracing agent can be a marker of unstable carotid plaque which can lead to cerebral ischemic

stroke. An investigation after PET has been revealed that the relationship between the uptake of tracer and the chances of recurrent stroke. This enabled better identification of patients who need revascularization of the artery. Another tracer Pittsburgh compound B has been used to investigate the formation of β-amyloid plaques after cerebral ischemic stroke; however, no correlation has been found between the ischemic attack and the accumulation of β-amyloid.

It has been extensively reported that ischemic stroke leads to the microstructural changes such as the disruption of blood-brain barrier which could further affect the other regions of the brain including penumbra and core. As such changes could not be investigated and monitored by MRI, so unique PET tracer such as ^{18}F-THK-5351 enabled researchers to monitor and investigate the aberrations that occur owing to cerebral ischemia and related complications (Harada et al. 2016; Huang et al. 2018). Monoamine oxidase B enzyme expressed in astrocytes in neuroinflammation condition readily binds with tracers and serves as a better approach to study (Huang et al. 2018). Therefore, all these advanced imaging techniques have been employed to monitor and perform imaging of real-time disease conditions in the brain such as cerebral ischemia and brain tumors. The use of nanoparticles along with these imaging techniques makes this a suitable approach to diagnose different states of diseases.

3.6 Limitations and Prospects

Regardless of the recent advancements in the field of imaging and diagnosis of cerebral ischemia which have helped a lot of patients, still, there is a huge gap that requires current consideration for improved and better imaging of cerebral ischemia and related complications. However, currently, MRI and CT are considered as gold standards for imaging. Although, these two techniques provide quick diagnosis and imaging with good resolution and better tissue permeation depth, therefore, these techniques could be used in an initial assessment of patient's conditions; however, the use of these techniques is limited due to certain issues.

As CT is readily available at a lower cost as compared to MRI, but due to its low contrast imaging, radioactive contrast agents have to be administered in the patients with X-ray radiation emitted/generated from the instrument, thereby, increasing radiation exposure could be dangerous. Additionally, CT is not a suitable technique to determine the actual damage as it is a semiquantitative way of imaging the tissue. Whereas, in the context of MRI, it endows higher spatial resolution with greater sensitivity and better tissue penetration, with minimal exposure of radiations as compared to CT. Regrettably, due to its high cost and poor contrasting ability in brain ischemia, the use of MRI is restrained for imaging cerebral ischemia.

Apart from CT and MRI, there are more techniques which have been discussed in this chapter, i.e., PET and SPECT that enable fast imaging with amazing tissue penetration to obtain the quantitative data to investigate the disease condition. However, these techniques are not quite common as compared to CT and MRI, due to their high costs, limited availability, and higher exposure to the radiations as

compared to CT and MRI. Additionally, other limitations that restrict the use of these techniques in the imaging of cerebral ischemia are poor spatiotemporal resolution and problem in the determination of results. There is still a technique which is in underway/development and can circumvent the current limitation in the imaging and diagnosis is MPI. Due to certain advantages such as excellent resolution, low emission of radiations, potential to quantify minute range, fast imaging, and no barrier in penetrating the tissue make this technique suitable and promising successor for the imaging and diagnosis of cerebral ischemia. Another technique that could be successfully used for imaging and diagnosis of brain ischemia is IVM. Nevertheless, its unbeaten excellent resolution for imaging at the cellular level could provide a lot of shreds of evidence that no other technique could provide, but due to many limitations provided by this technique its use has been restricted in the investigation of brain tissues. But, to circumvent these limitations, the combination of various techniques could be used such as MRI/CT, PET/CT, and many more.

An advanced, fast, accurate, low cost, and reliable technique is indispensable for improved imaging and diagnosis of cerebral ischemia. Therefore, there is a requirement to foster the advancement of nanoagents as well techniques that are employed in imaging the disease condition. But the development of MPI may improve and circumvent the current limitations to some extent. Additionally, the use of nanoagents in imaging could endow a lot of information about the disease as the administration of nanoagents could improve the circulation transit time and enhance the targeting ability that ultimately increases the imaging potential of contemporary imaging techniques by removing the potential side effects. We anticipate that in upcoming/next coming years the development of advanced imaging techniques and instruments that restricts the use of ionizing energies and any radioactive or radiotracer and endow an advanced therapeutic and imaging window with fast, accurate, and economical imaging of cerebral ischemia for all types of patients will hold strong grounds/will exert significant effects.

3.7 Conclusion

Cerebral ischemia is considered one of the deadly diseases and the biggest challenge at this time. Even though various nanotechnology approaches have been investigated in the last few years, but still, there is a gap in the potential therapeutic approach for cerebral ischemia. The narrow therapeutic window, presence of barriers, and side effects after the ischemia attack occurred during reperfusion also restrict these treatments. Additionally, these limitations restrict the use of techniques and could not endow sufficient information regarding patients' problems. However, the employment of smart nanotherapeutics could deliver the therapeutic agent and ameliorate neuronal damage due to subsequent inflammation. Also, the combination of nanotherapeutics and nano-imaging agents could endow extensive information on the complications due to cerebral ischemia. There has been a lot of advancement done in the field of nano-therapeutics and imaging modalities; however, there is still a scope for improvement. Finally, it is obvious that the multimodal nano-theranostics

can enable multiple approaches to be performed at a time for imaging as well as therapy of cerebral stroke.

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4 Cell-Mediated Neurorestorative Mechanisms Underpinning Beneficial Effects in Ischemic Stroke

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Abstract

Stroke is the most common cause of death and disability throughout the world. The development of promising stem cell-based interventions with the potential to result in significant and possibly complete recovery of brain function is underway. However, the brain's ability to self-repair is severely restricted during the acute and chronic phases of a stroke. Several preclinical and clinical studies on stem cells as therapeutics have been published in the last decade. These studies have contributed to our understanding of cell therapy by demonstrating that it is safe and moderately effective. The replacement of cells is no longer considered the primary mechanism of action for cell therapy treatments, but rather a combination of therapeutic mechanisms may provide neuroprotection in stroke patients. This chapter aims to talk about how paracrine effects, neurogenesis,

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synaptogenesis, and apoptosis inhibition, and endogenous circulating or site-specific stem cells can help an ischemic brain.

Keywords

Stroke · Neuroprotection · Cell therapy · Paracrine effects · Neurogenesis · Synaptogenesis

4.1 Introduction

Ischemic heart disease and stroke killed 152 million people in 2015 (range: 0–-156 million) (Group GBDNDC). GBD 2015, a study of the global burden of disease, injuries, and risk factors, found that there has been a steady shift away from communicable diseases and toward non-communicable diseases such as stroke and heart disease. Each year, at least one stroke survivor out of every six will have another stroke within the next 5 years. (Benjamin et al. 2017). This effect is most likely the result of global population growth and aging (Mortality GBD 2016). In the previous two decades, the stroke burden has risen among people aged 20–64, particularly in low- and middle-income countries. The global incidence of stroke has been increased by 25% among those under 65. Because of Asia's large population, stroke is a far more severe problem in Asia than in any other region of the world. Asia has a higher stroke mortality rate than Western Europe, the Americas, or Australasia, and Eastern Europe has the same rate (Katan and Luft 2018; Venketasubramanian et al. 2017; Jha et al. 2008). In some regions, such as Russia, China, and India, an epidemic of cardiovascular risk factors among young adults, such as hypertension and diabetes, has increased the stroke burden among younger populations (Feigin et al. 2017; Norrving and Kissela 2013; de los Rios et al. 2012; Hu et al. 2012; Zaridze et al. 2009).

Strokes can be classified as ischemic or hemorrhagic. Although ischemic stroke is more common (87% of all strokes), a hemorrhagic stroke results in a more significant number of deaths and loss of disability-adjusted life years. Because of its high prevalence, the former has become the focus of all scientific and laboratory research. For many years, thrombolytic therapy has been recognized as the most effective treatment for ischemic stroke. Due to the limited clinical time window of 3–4.5 h, thrombolytic therapy is only helpful for a small proportion of patients (Raza et al. 2013; National Institute of Neurological Disorders and Stroke 1995), necessitating the development of novel therapies.

Stem cell therapy has been identified as a viable therapeutic strategy capable of significantly improving patients' recovery from ischemic stroke. Preclinical research suggests that stem cells may reduce neurological deficits caused by a cerebral stroke. Some of these cells have also been tested in clinical trials, with mixed results. Notably, recent research has shown that replacing dead cells or badly damaged brain tissues via stem cell transplantation is far from feasible. Recently, we discovered that both hDPSCs and human mesenchymal stem cells (hMSCs) die in an

oxidative and inflammatory (micro-) environment (Prakash et al. 2021; Ahmad et al. 2019). Acute inflammation/immune response, trophic factor removal, oxidative stress, excitotoxicity, hypoxia, or anoikis, for example, are all common causes of stem cell death in the first few days following transplantation. Cell therapy is hampered by improper cell fate control and cell engraftment after transplantation, in addition to poor survival. As a result, the need to develop new treatment techniques that maximize the effects of cell therapy remains an open question.

Numerous studies have been initiated to gain a better understanding of the mechanisms underlying transplanted cells' restorative abilities in the ischemic brain. However, most published research studies assessed stem cells' efficacy in promoting functional healing through behavioral testing. Regardless of their undeniable importance, behavioral studies do not shed light on the molecular mechanisms underlying observed functional outcome improvement. A series of studies spanning several years indicates that stem cells, in addition to neural replacement, contribute to recovery in ischemic brains by facilitating angiogenesis, synaptogenesis, immunomodulation, and apoptosis inhibition. This chapter provides an overview of potential neural recovery mechanisms in conjunction with stem cell therapy.

4.2 Stem Cell Types for Stroke Recovery

Until the mid-nineteenth century, neural regeneration was believed to be impossible. In this regard, neurodegenerative diseases and injuries were deemed incurable and only symptomatic treatment was offered. Even today, none of those above diseases have an effective treatment. Stem cell therapy is viewed as a potentially transformative method for treating neurological disorders, including stroke. Numerous cell types have been investigated as potential donors, including bone marrow mononuclear cells (BMMNCs), bone marrow/adipose-derived stem/stromal cells (BMSCs/ADMSCs), dental pulp stem cells (DPSCs), umbilical cord blood cells (hUCBCs), hematopoietic stem cells (HSCs), neural stem cells (NSCs), olfactory ensheathing cells, and porcine fetal cells. We will discuss a few different stem cell types concerning stroke treatment in this section.

Mesenchymal Stem Cells MSCs (mesenchymal stem cells) are pluripotent adult stem cells that can self-renew (Urrutia et al. 2019). They were initially discovered in bone marrow (Friedenstein et al. 1969) but have been discovered in various other tissues and organs since then, including umbilical cord tissue (McElreavey et al. 1991), umbilical cord blood (Erices et al. 2000), adipose tissue (Zuk et al. 2002), skin (Toma et al. 2005), and pancreas (Seeberger et al. 2011). CD73, CD90, and CD105, while being negative for CD34 and CD45, is a minimal immunological positive criterion for a cell to be classified as MSCs. MSCs, in particular, can transdifferentiate into neural precursors and/or mature neurons, promoting neuroprotection and neurogenesis. Additionally, MSCs exhibit immunomodulatory properties, produce trophic factors that aid tissue repair and regeneration, and differentiate into various cell lineages, including neurons and glial cells (Urrutia

et al. 2019). MSCs are often used in cell therapy and tissue repair for many diseases, including stroke research.

Human Umbilical Cord Blood Cells Human umbilical cord blood cells (hUCBC) are widely used as an abundant and ethically acceptable source of regenerative stem cells. hUCBC is widely available and only rarely contaminated with infectious agents like cytomegalovirus or Epstein–Barr virus (Rubinstein et al. 1993). A high proportion of $CD34^+$ and $CD105^+$ cells in hUCBC indicates that it has a high capacity for regeneration. hUCBC is also a source of cells with low oncogenic potential and longer telomeres (McGuckin and Forraz 2008). It is worth noting that because they are less sensitive to variations in human leukocyte antigen (HLA) molecules, hUCBCs are less likely to be rejected by the immune system (Danby and Rocha 2014). hUCBCs are becoming a more appealing option for long-term neurological restorative recovery after stroke in rodents, according to recent reports (Chen et al. 2001).

Dental-Derived Stem Cells Our teeth are the most natural and noninvasive source of stem cells. Dental stem cells (DSCs) have become an appealing source for cell-based neurorestorative therapies due to their neural crest origin, ease of harvest, accessibility, moral suitability, and ability to differentiate into the neurogenic lineage. Another advantage of DSCs is that they are free of ethical concerns, which is a disadvantage associated with cell types such as induced pluripotent stem cells and other adult stem cells (Yalvac et al. 2009). DSCs can develop into various brain cell types, including neurons and glia, demonstrating their neurogenic potential (Rafiee et al. 2020; Zainal et al. 2013). It is expected that the transplanted DSCs will differentiate and integrate into the damaged CNS (Raza et al. 2018; Leong et al. 2012). DSCs have shown increased neurogenesis (Akiyama et al. 2012). These cells also impact endogenous stem cell recruitment and neurosphere technology (Huang et al. 2008). Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells, and stem cells from the dental apical papilla (SCAPs) are the names given to stem cells based on their location in the tooth (Raza et al. 2018). They have visible protein markers such as CD90, CD73, CD105, and CD44, but not CD34, CD31, or CD45 (Raza et al. 2018). DSCs are formed from the neural crest (Komada et al. 2012). DSCs are a type of mesenchymal stem cell, and as such, they have a high immunosuppressive potential (Alipour et al. 2013; Kerkis et al. 2008). DSCs have significantly higher clonogenicity and ex-vivo proliferative capability than MSCs (Akiyama et al. 2012; Shi et al. 2005); they are less prone to malignancy (Wilson et al. 2015) and can produce enough cells for cell therapy. DSCs show delayed cellular senescence (Ren et al. 2016), which could be attributed to increased genes associated with growth factors (Kang et al. 2016). In recent years, the two subtypes of DPSC and SHED have been extensively studied and employed to investigate the neurological restorative measures of cell integration.

Neural Stem Cells Neural stem cells are multipotent cells that can self-renew and proliferate indefinitely, giving rise to offspring cells that develop into neurons, astrocytes, and oligodendrocytes. Nestin and SOX2 are two commonly used neural stem cell markers. Nestin is a useful marker because it is absent from nearly all mature CNS cells while it is highly expressed in CNS stem cells. SOX2 is highly expressed in the developing CNS's neuroepithelium and is thought to play an essential role in neural stem cell proliferation and differentiation. Products for studying proteins expressed on the cell surface of these cells, such as CD133, Pax6, N-Cadherin, Musashi-1, Musashi-2, and Frizzled-9, are available in addition to intracellular molecules. NSCs have several advantages, including the ability to self-renew, have low immunogenicity, have good histocompatibility, and differentiate into three distinct types of nerve cells to maintain and repair damaged brain tissue. As a result, NSCs are an effective tool for treating nervous system diseases and serving as an active natural resource.

The mature mammalian central nervous system consists of three major cell types: neurons, astrocytes, and oligodendrocytes (CNS). The neuroectoderm is induced through mammalian embryogenesis, which later folds and forms the neural tube. Neuroepithelial progenitor cells (NEPs), the earliest neural stem cell type, develop and reside within these neural structures (Kriegstein and Alvarez-Buylla 2009; Temple 2001). As the nervous system develops, a variety of neural stem/progenitor populations arise. During brain development, NSCs transition to asymmetric division cycles generates lineage-restricted progenitors. With these findings, a new era of study on the vast amount of untapped potential in these cells has begun.

4.3 Mechanisms Underlying Neuroprotection in Stroke

Neurodegeneration characterizes the brain insult (possible loss of neuronal architecture and function). Because neuroprotective techniques have failed to save or replace injured CNS tissues, the focus has shifted to neurorestorative therapy. Several studies have investigated the mechanisms that aid in recovery (Fig. 4.1); some of these studies are summarized below:

4.4 Cell Replacement

Cell replacement has long been considered the primary salvage strategy. Continuous research is being conducted to understand better how stem-cell therapy restores function in neurological illnesses via cell replacement. Cell replacement can be accomplished in two ways: one using endogenous stem cell delivery and the other using external stem cell delivery. The endogenous method's goal is to promote the formation, mobilization, and stability of stem cells within a person. Newly generated cells from the subventricular zone (SVZ) and the dentate gyrus (DG) are capable of replacing dying neurons in the post-ischemic brain by directly migrating to sites of

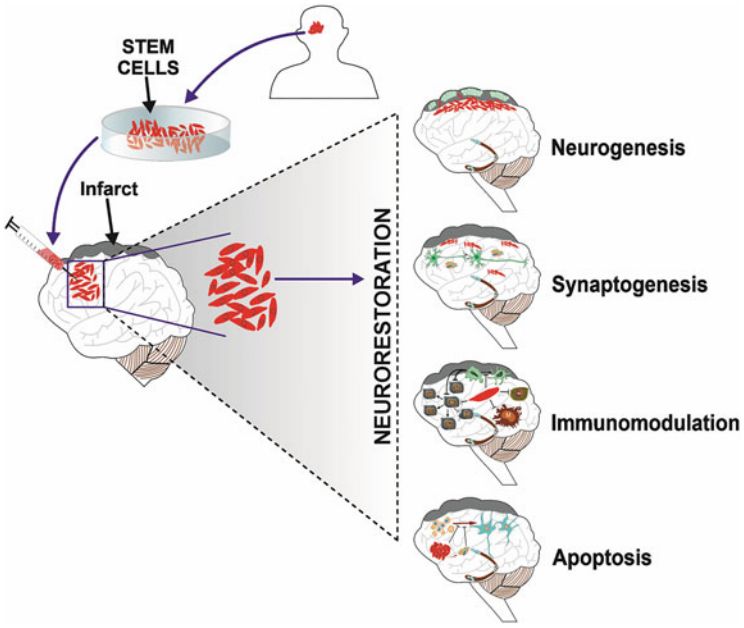

Fig. 4.1 A schematic diagram depicting the mechanistic processes underlying stem cell-induced neurorestoration in an ischemic stroke brain. Transplanted stem cells may initiate a cascade of restorative events via cell replacement, paracrine secretion, trophic factors, angiogenesis, immunomodulation, and apoptosis inhibition

injury (Kojima et al. 2010). In response to experimental stroke, endogenous NSCs multiply and migrate (Kokaia and Lindvall 2012; Thored et al. 2006). According to reports, the direction of regeneration is influenced by the endogenous neurogenic reaction (Jin et al. 2010; Wang et al. 2012). However, as people continue to suffer from varying degrees of post-ischemic damage physical and cognitive morbidity, it is clear that our own endogenous healing mechanisms are far from optimal (Wolfe et al. 2011; Kumar 2010). To replace the lost cells, exogenous stem cell replenishment could be used. Because of their innate ability to move (phototropism), these exogenous stem cells can function as a virtually infinite source of new brain cells and can incorporate into ischemic tissue (Jin et al. 2003; Zhang 2003). The exogenous strategy entails transferring NSCs from a single source (Hermann et al. 2014; Bacigalupi et al. 2009).

HSC, MSC, DPSC, UCBC, and NSCs are all widely accepted among other cell replacement options because they differentiate and integrate into the recipient tissue after transplantation (Fig. 4.2). In addition to neurons, grafts can differentiate into glial cells such as astrocytes, microglia, and oligodendrocytes in vivo (Rosenblum et al. 2012). According to numerous preclinical studies, exogenous NSC transplantation significantly replaces damaged tissues and can be used to treat a variety of neurological diseases (Chen et al. 2016; Li et al. 2014; Kwakkel et al. 2004). For example, NSCs overexpressing the SUMO E2-conjugase Ubc9, when transplanted

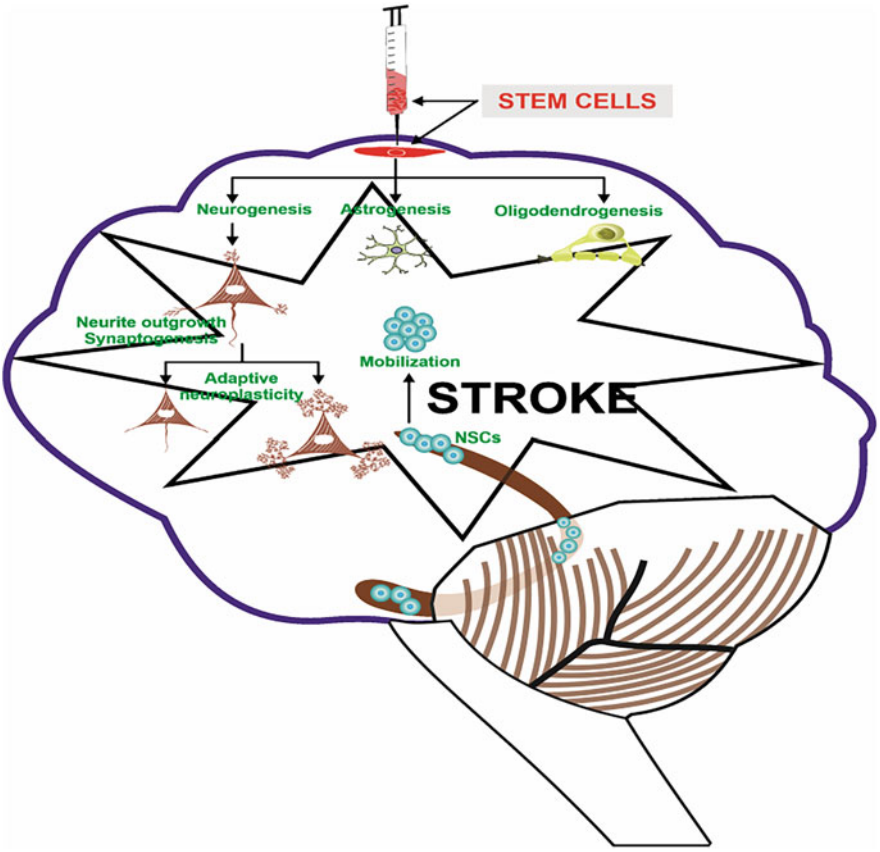

Fig. 4.2 The neuroplasticity mechanism of stem cells in stroke recovery is mediated by transdifferentiation

into mice with transient middle cerebral artery blockage, lead to better survival and neuronal differentiation (Bernstock et al. 2019). Exogenous DSCs have been shown to promote endogenous neurogenesis, just like NSCs (Xiao et al. 2017). When DPSCs are implanted in a mouse's hippocampus, they affect the mobilization of endogenous neural stem cells. Long-term transplantation results in the proliferation of newly generated neurons at the graft site, resulting in neural progenitor cells (NPCs) and neurons (Huang et al. 2008). Similarly, in the ischemic brain, SHED-conditioned media aided in the migration of lost neurons and the differentiation of endogenous NPCs (Inoue et al. 2013). MSCs, like NSCs and DPSCs, can replace dying brain cells. The results from Azizi et al. (1998) show that about 20% of human BMSCs transplanted into a rat brain are viable after 72 days and show neuronal phenotype. In addition, stem cells have been seen migrating to the damaged area (Shichinohe et al. 2007). Even though engrafted stem cells can give rise to mature neuronal lineage cells with electrical properties (Oki et al. 2012; Daadi and Steinberg

2009), the overall number of survived engrafts is significantly lower of neurons lost due to stroke. The rate of neuronal differentiation of engrafted stem cells after a stroke has been observed to vary between 10% and 60% in most studies (Daadi and Steinberg 2009; Buhnemann et al. 2006), which is significantly less than what is required. As this ability may not be sufficient to achieve a meaningful recovery, improving the effect may be an effective stroke therapy strategy.

Rather than focusing on the direct replacement of brain cells, the new hypothesis focuses on additional reparative mechanisms such as neurogenesis, bystander effect, new synapse creation, and so on. We'll take a look at a few of them here:

4.5 Neurogenesis

Numerous independent research studies have confirmed that neurogenesis occurs even in adults (Wang and Jin 2015). Neurogenesis describes the creation of new functional neurons from neural stem/progenitor cells (NSCs/NPCs). Neurogenesis occurs in two distinct locations in the intact brain: the SVZ of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (SGZ). Ischemic insults induce neurogenesis in the brains of rodents (Table 4.1) and humans (Cramer and Nudo 2010; Chang et al. 2007; Thored et al. 2006; Jin et al. 2001, 2006), demonstrating that the adult brain can repair itself. Endogenous NSC proliferation is the first stage of stroke-induced neurogenesis (Tang et al. 2009). Cortical ischemia has recently been shown to influence neuroblast proliferation in the SVZ (Liang et al. 2019). Furthermore, exogenous stem cell therapy has been shown to induce neurogenesis in the ischemic brain. Shiota et al. (2018) showed that transplanting mesenchymal stem cells caused neuronal progenitors to proliferate and migrate earlier. MSC transplantation has been shown to increase oligodendrocyte progenitor proliferation in a cerebral ischemia model (Yu et al. 2018), indicating further that exogenous stem cell transplantation affects endogenous stem cells. Several preclinical studies indicate that NSC transplantation is an effective treatment for ischemic stroke due to increased neurogenesis and angiogenesis (Sinden et al. 2012; Miljan and Sinden 2009). These findings suggest that the benefits of stem cell therapy are positively related to endogenous neurogenesis, which can be induced naturally or through exogenous stem cell supplementation in experimental ischemic stroke.

4.6 Paracrine Signaling or Bystander Effect

Extensive stem cell graft-to-host contact following stem cell transplantation in experimental stroke results in significant trophic/plasticity benefits as well as favorable immunomodulatory effects, in addition to the replacement of damaged cells (Bacigaluppi et al. 2016) (Table 4.2). In this sequential interaction, trophic factors are created in the CNS that modulate cells and their surrounding microenvironment, promoting tissue homeostasis (Pluchino and Cossetti 2013; Martino et al. 2011).

Table 4.1 Neurogenesis in stroke

S. no.	Stem cell types	Host	Outcome	References
1.	BMSCs	Wistar rats	1. Contralesional axons sprouted 2. EGFP-positive pyramidal neurons increased in the infarct area 3. Reduction in ipsilateral thresholds 4. Enhanced functional outcome	Liu et al. (2008)
2.	BM-MSCs	Sprague–Dawley rats	1. Improved neurological function 2. BMSCs combined with TMP enhanced SDF-1 and CXCR4 expression in ischemic regions 3. BMSCs combined with TMP promoted angiogenesis and neurogenesis	Li et al. (2019)
3.	MSCs	Sprague-Dawley rats	1. Enhanced neurogenesis and angiogenesis	Liu et al. (2018)
4.	hNSCs	Sprague-Dawley rats	1. Enhanced angiogenesis	Ryu et al. (2016)
5.	AD-MSCs	Sprague-Dawley rats	1. Enhanced neurogenesis and angiogenesis	Ryu et al. (2019)
6.	MSCs	C57BL6 mice	1. Enhanced angio-neurogenesis 2. Enhanced motor co-ordination	Doeppner et al. (2015)
7.	BM-MSCs	Sprague–Dawley rats	1. CXCR4 expression decreased	Zhang et al. (2016)

A paracrine (or bystander) effect mediated by stem cells is a sort of communication in which the implanted cells' trophic factors alter the surrounding environment's molecular makeup and activate responses from resident cells. The neuronal population's growth, maintenance, repair, and survival are all dependent on the release of trophic factors by stem cells in the brain (Baraniak and McDevitt 2010; Tucker 2002; Schinder and Poo 2000). Several studies have shown that implanted cells can no longer be recognized on histological parts over time while still demonstrating stable and consistent functional improvement, implying a possible role for other neurorestorative mechanisms, which the cells attributed to paracrine effects (Li et al. 2021; Raza et al. 2018; Green et al. 2018; Bhasin et al. 2016). In recent years, the paracrine effect has been extensively studied, and almost all cell types have demonstrated the ability to secrete various growth secretions. In animal studies, for example, DSCs provide cytoprotection by secreting neurotrophic peptides that aid in neural repair and regeneration (Chang et al. 2014; Mead et al. 2014; Ishizaka et al. 2013; Arthur et al. 2009). In neurological disorders, after DSC transplantation, the tissue concentrations of vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF),

Table 4.2 Paracrine effects in stroke

S. no.	Stem cell types	Host	Outcome	References
1.	BMSCs	Male Wistar rats	1. Increased TRPC6 and BDNF protein expression 2. BM-L-C6 reduced neuronal death	Li et al. (2019)
2.	MSCs	Female Sprague–Dawley (SD) rats	1. Reduction in calcineurin in the peri-infarct area 2. Increase in GSH levels was observed in MSCs treated 3. Improved functional outcome	Saraf et al. (2019)
3.	Human NSCs	Adult, male NMRI-nu mice	1. A rapid decrease in functional connectivity strength 2. Decrease in functional sensorimotor network 3. Fiber density increases between the cortex and white matter regions	Green et al. (2018)
4.	hNPCs and iPSCs	Adult Sprague–Dawley rats and marmosets	1. Improved functional recovery	Surugiu et al. (2019)
5.	MSCs	Sprague–Dawley rats	1. OGD treatment induced decreased viability of BV2 cells in a time-dependent manner 2. Cell apoptosis also showed that BMSCs-CM significantly inhibited OGD/R-induced apoptosis 3. Hypoxia preconditioning of BMSCs increased production of exosomes	Yu et al. (2020)

ciliary neurotrophic factor, glial cell-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT3) have been observed increased significantly (Fuji et al. 2015; Chang et al. 2014; Sakai et al. 2012). In vivo, when DPSCs interacted with the developing nervous system, neuroplastic changes were observed that were attributed to DPSC secretion. The authors demonstrated that CXCR4 receptors are key in promoting the chemo-attraction of avian trigeminal ganglion axons to implanted DPSCs (Nosrat et al. 2001). Similarly, when NSCs were grafted into a rodent model of stroke, significant trophic/plasticity resulted in extensive stem cell graft-to-host communication following transplantation, in addition to the replacement of damaged cells (Bacigaluppi et al. 2016). Duan et al. (2017) discovered a significant increase in BDNF, GDNF, VEGF, and interleukin 6 (IL-6) expression in animals grafted with labeled and unlabeled MSCs, with a peak at 2 weeks and a gradual decrease at 4 and 8 weeks after transplantation. This dynamic shift in secretion occurred at the same time that the number of surviving stem cells started to decline. To summarize, the findings suggest that one of the primary mechanisms of action of stem cells in stroke therapy is paracrine, with neuroprotective, antigenic, and immunoregulatory effects. While more research into the specific mechanism underlying the paracrine impact is required, the current findings are entirely consistent with numerous other findings

(Bakondi et al. 2009; Stroemer et al. 2009; Locatelli et al. 2009; Chopp and Li 2002; Caplan and Dennis 2006).

4.7 Immunomodulation

Inflammation has always been linked to stroke pathogenesis (Jayaraj et al. 2019; Meng et al. 2019) (Table 4.3). Ischemia-induced DAMP production by neuronal and glial cells activates astrocytes and microglia, leads to the production of pro-inflammatory cytokines and chemokines, disrupts the blood-brain barrier (BBB), and results in the infiltration of peripheral blood leukocytes into the infarcted area, exacerbating tissue damage (Dabrowska et al. 2019). In other words, microglia and monocytes can produce anti-inflammatory cytokines and protect injured nervous tissue. Immunosuppression may reduce acute tissue damage and promote endogenous stem cell migration and survival (Erlandsson et al. 2011). Local inflammation attracts stem cells to the infarct, inducing growth factors (Kelly et al. 2004). This demonstrates the limitations of approaches based on complete inflammation suppression.

According to a recent paradigm shift, stem cells' beneficial properties may be due in part to their immunomodulatory functions. A spleen-dependent neuroprotective effect was observed after systemic administration of neural progenitor stem cells (NPSCs) to ischemic rats. In summary, intravenous injection of NPSCs 2 h after stroke improved functional recovery, decreased infarct size and edema, and decreased brain inflammatory infiltration. Cytokine analysis in the brain and spleen revealed decreased pro-inflammatory expression of TNF, IL-6, and NF-k. A large number of NSPCs were also found in the spleen; splenectomy eliminated the edema and immune cell infiltration in the brain (Lee et al. 2008). Moreover, iPSCs have been shown to reduce innate immune cells' inflammatory reactivity and polarization and attenuate circulating pro-inflammatory mediators in the context of a focal ischemic stroke. When iPSC and fibrin glue were infused into an experimental stroke model, they significantly reduced interleukin (IL)-1, TNF-, IL-2, and IL-6, as well as iNOS expression in the lesion cortex. Furthermore, compared to the MCAO-only group, IL-4 and IL-10 protein levels were significantly higher in the iPSC and fibrin-treated groups (Chen et al. 2010).

Transplanted cells secrete cytokines that directly limit T-cell activation, limit leukocyte infiltration into the brain and local inflammation, and modify the immunological response, resulting in immunomodulatory benefits following stroke. Several studies have proven that MSCs affect the immune system (Gouveia et al. 2015; Uccelli and de Rosbo 2015; Ooi et al. 2010). The immune response associated with cerebral ischemia has been shown to be modulated by intracerebral MSC transplantation. They secrete factors like TGF-, which inhibit MCP-1 secretion and limit CD68+ cell infiltration into damaged tissue (Yoo et al. 2013; Yasuhara et al. 2009). MSCs can also directly inhibit T lymphocyte and microglial cell proliferation and change the cytokine secretion profile of dendritic cells and monocytes. DSCs, like MSCs, have potent immunomodulatory properties. DSCs modulate immune

Table 4.3 Immunomodulation in stroke

S. no.	Stem cell types	Host	Outcome	References
1.	ESCs and iPSCs	Animal models and human patients	1. Improved functional outcomes in patients	Dabrowski et al. (2019)
2.	BM-MSCs	Human	1. Decrease in cell death in the penumbra area	Hammadi and Alhimyari (2019)
3.	hUCBCs	Sprague Dawley rats	1. Downregulating	Shiao et al. (2019)
4.	MSCs	Wistar rats	1. Unregulated TLR2 expression 2. Increased Bax expression and IL-10 release rate 3. Downregulated TLR2/NFκB signaling	Gu et al. (2015)
5.	hMSCs	Wistar rats	1. Improved angiogenesis 2. Increased VEGF and BDNF expression in astrocytes 3. Enhanced functional recovery	Zhang et al. (2017)
6.	MSCs	Sprague-Dawley rats	1. Reduced apoptosis 2. Increased cell proliferation	Zhou et al. (2015)
7.	BM-MSCs	Sprague-Dawley rats	1. Increased VEGF and BDNF expression 2. Enhanced SDF-1/CXCR4 expression, thereby, enhanced migration	Li et al. (2019)
8.	NSCs	Sprague-Dawley rats	1. MHP36 murine neuroepithelial stem cells showed low immunogenic properties	Modo et al. (2002)
9.	Neural stem cells	C57/BL6 mice	1. Enhanced endogenous stem cell mobilization and recruitment to the injury site 2. Enhanced functional improvement	Erlandsson et al. (2011)
11.	MSCs	C57BL/6 mice	1. No effect of MSCs administration (interleukin-6, tumor necrosis factor-a, interferon-c, monocyte chemoattractant protein-1 remained unchanged post-MSC infusion)	Scheibe et al. (2012)

responses by secreting a wide range of trophic factors essential for injury repair (Blesch et al. 1998). DPSCs, for example, can inhibit T and B cells while enhancing natural killer (NK) cell resistance (Li et al. 2014; Yang et al. 2009). When CD4+ T cells and DPSCs were co-cultured, the T cells expressed a high amount of Treg (Li et al. 2014). This interaction may alter the expression of transduction signaling systems, boosting the suppression of lymphocyte and natural killer cell production,

for example, SHEDs reduced T cells and promoted regulatory T cells (Tregs) in an experimental autoimmune encephalomyelitis model (Rossato et al. 2017). Overall, it is reasonable to believe that stem cells' immunosuppressive capacity provides a distinct advantage in the clinical management of ischemic stroke.

4.8 Synaptogenesis

Synaptogenesis, in order to achieve improved communication and connectivity between neurons, is a key aspect for neurological improvement in stroke recovery (Chen et al. 2003). The process by which neurons form synapses is known as synaptogenesis. Synaptophysin is a protein found in the presynaptic vesicles of all nerve terminals. It is frequently used as a marker of synaptogenesis (Stroemer et al. 1995). Neuroplastic changes in DPSCs were obtained using an avian embryonic model system in which engrafted DPSCs produced unknown neurotrophic factors that determined axon direction within the beneficiary host nervous system (Arthur et al. 2009). The release of neurotrophic factors was found responsible for maintaining neuronal circuit integrity and plasticity (Chiu et al. 2017; Lee et al. 2016). Cui et al. (2012) discovered combining hUCBC with simvastatin improved synaptic plasticity, axonal and neurite outgrowth, and BDNF expression. According to Ding et al. (2013), stem cell therapy stimulates synaptogenesis by increasing synaptophysin immunoreactivity in the ischemia border area following transplantation (Ding et al. 2013). Undamaged neurons in the peri-infarct area are stimulated to sprout and form new synaptic connections due to hypoxia (Stroemer et al. 1995). As per the above findings, stem cells may induce functional recovery by altering the synaptogenic pathway (Table 4.4).

4.9 Apoptosis

In the immediate aftermath of the initial ischemic stroke, a critical secondary cell death mechanism leading to neurological damage is the upregulation of endogenous neuroinflammatory processes, which activate the programmed cell death pathway and destroy hypoxic tissue local to the area of the insult (Wang et al. 2020). Stem cells may protect ischemic brains from apoptosis. MSCs, for example, promote endogenous neurogenesis by recruiting primary stem cells from the SVZ and DG to the site of injury and decreasing the rate of apoptotic insult in the primary lesion's penumbral zone (Li et al. 2016). When BM-MSCs were co-cultured in OGD-injured neuron models, they reduced apoptosis and necroptosis rates by downregulating necroptosis-related receptor-interacting protein kinase 1 and 3 and deactivating caspase-3, an apoptosis-related enzyme (Kong et al. 2017). According to another report, pro-apoptosis factor expression was suppressed in response to ER stress with the addition of AD-MSCs. In contrast, anti-apoptosis factor expression was raised (Chi et al. 2018). Likewise, intravenous infusion of autologous endothelial progenitor cells (EPCs) after MCAO in rabbits resulted in functional improvement, fewer

Table 4.4 Synaptogenesis in stroke

S. no.	Stem cell types	Host	Outcome	References
1.	MSCs	Sprague–Dawley rats	1. MSC infusion reduced lesion volume 2. Induced synaptogenesis MSC infusion, but the effect was greater	Sasaki et al. (2016)
2.	hNSCs	Wistar rats	1. Undifferentiated hNSCs displayed a greater proliferation 2. Upregulated expression 3. Improved functional recovery	Abeysinghe et al. (2015)
3.	iPSCs	Nude rats	1. Cells maintained a higher input resistance 2. Strong propensity for GABAergic differentiation	Avaliani et al. (2014)
4.	NSCs	Sprague-Dawley rats	1. Decreased expression of nestin and GFAP 2. Enhanced expression of Map2 3. PSD95 increased	Zhang et al. (2006)
5.	NSCs	Sprague-Dawley rats	1. Enhanced NSCs proliferation and neuronal differentiation 2. Enhanced synaptogenesis	Wang et al. (2019)
6.	hNSCs	Sprague-Dawley rats	1. Enhanced synaptogenesis 2. Improved cognition	Ager et al. (2015)
7.	hNPCs	SCID/Beige	1. Increased synaptogenesis 2. Promotes functional recovery	Vonderwalde et al. (2020)

apoptotic cells, more microvessel density in the ischemic peri-infarct, and a smaller infarct area (Chen et al. 2008). These findings imply that stem cells exert their influence, at least in part, via their anti-apoptotic activity.

4.10 Conclusion

Transplantation research in ischemic stroke should aim to understand the molecular and functional mechanisms underlying the benefits. Stem cells have the potential to overcome current barriers in the field of neurodegeneration via a variety of mechanisms, including cell replacement, bystander effect, synaptogenesis, immunomodulation, and inhibition of apoptosis. As a result, all neurorestorative treatments should be considered in stroke and related disorders when using stem cells.

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5 Induced Pluripotent Stem Cells for the Treatment of Neurodegenerative Disease: Current and Future Prospects

Ankita Kumari, Archana Chaudhary, and Rizwanul Haque

Abstract

Neurodegeneration is a broad category for the progressive and unregulated incremental deterioration of motor neuron functionality or mortality of neurons in the central nervous system (CNS). The number of patients with neurodegenerative diseases is expanding substantially around the world. However, the underlying mechanisms of their progressive nature are not yet fully elucidated and for their early detection, there are no biomarkers available. Uses of post-mortem tissues or animal models like transgenic mouse model or knockout model have their own limitations as it is not feasible to analyze patient neurons prior to degeneration as they are not able to adequately recapitulate disease phenotype. Thus, intense research is imperative to discover pathways of disease advancement in an exertion to identify the molecular therapeutic for restorative medication. Induced pluripotent stem cells (iPSCs) extracted from patients can be transformed into disease-specific neurons, offering an unprecedented opportunity toward in vitro research and the emergence of targeted therapies. In spite of numerous challenges, stem cell therapy explores one potential paradigm change by flipping from neuroprotective treatments to neurodegeneration/neurorestorative modalities for neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). Induced pluripotent stem cells (iPSCs) are marked by their capability to self-renew indefinitely and the ability to transform into any kind of cells by reprogramming the human adult somatic cells through the introduction of non-viral and non-integrating methods or by innovative gene-editing strategies

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for generating genetically modified lines and are independent of any ethical issues as no destruction of embryo involved. The innovative iPSC approaches will have the ability to upgrade the knowledge of pathogenesis incredibly and encourage the advancement of innovative therapies as a beneficial approach for regenerative medicine to substitute diseased or damaged tissues.

Keywords

Induced pluripotent stem cells (iPSCs) · Stem cell therapy · Embryonic stem cells (ESC) · Transcription factors · Somatic cell reprogramming · Neurodegenerative disease · Cell replacement/regenerative therapy

5.1 Introduction

Human embryonic stem cells originating from the inner cell mass of embryos at the blastocyst stage have the extraordinary potential of indefinitely self-renew thereby retaining the capacity to yield all forms of cells in the human body (Thomson et al. 1998). Stem cells have the ability to differentiate into all the 3 germ layers – endoderm, mesoderm, and ectoderm and are characterized by their capabilities of self-renewal by which they can undergo several cycles of cell division while sustaining an undifferentiated phase and potency, i.e., the potential to produce cells of many lineages (Lowry and Plath 2008). These crucial principles of embryonic stem cells are also inherited by induced pluripotent stem (iPS) cells but are alternatively induced by reprogramming somatic cells through the controlled expression of core transcription factors. Following the very first study by Yamanaka and his colleagues in 2006 on the formation of induced pluripotent stem cells (iPSCs) from mouse fibroblasts, somatic cell reprogramming has created tremendous attention. In 2006, Takahashi, Yamanaka, and colleagues discovered that retrovirally introducing a cocktail of only 4 embryonic transcription factors, i.e., Oct3/4, Sox2, Klf4, and c-Myc, stable pluripotency could be induced in adult cells similar to embryonic stem cells (ESCs) from somatic cells of the mouse (such as fibroblasts) without any embryo. These cells were called induced pluripotent stem cells. iPSCs are produced artificially from somatic cells of specific individuals with known genotypes and detectable phenotypes and are relatable to progenitor cells in function therefore particularly appropriate to the modeling of diseases. iPS cells can be generated not only from fibroblasts but also from adult mouse hepatocytes and gastric epithelial cells and cells from human adult skin fibroblasts with the same four factors (Takahashi and Yamanaka 2006).

Co-transduction produced the first iPS cell lines with viruses expressing 24 distinct factors (Lowry and Plath 2008). Subsequent research reduced the requisite factors to four: Oct3/4, c-Myc, Sox2, and Klf4, with the most significant being Oct3/4. Oct3/4 expression is extremely peculiar to pluripotent stem cells, while the other aforementioned control dimensions are found in numerous different cells (Klf4 in the intestine, skin, skeletal muscle, and stomach; Sox2 in neural stem and progenitor

cells; c-Myc is ubiquitously expressed). In contrast, to produce iPS cells, any other member of the Oct family such as Oct1 or Oct6 cannot substitute Oct3/4. Klf4, on the other hand, can be substituted with Klf2 or Klf5, Sox2 with Sox1, and c-Myc with L-Myc or N-Myc. For ES cell pluripotency maintenance, Oct3/4 is strictly necessary. Suppression of Sox2 contributes to cell differentiation of ES, but this feature is preserved by the induced expression of Oct3/4. Mice deficient in Klf4 or c-Myc survive at birth, implying its pluripotency retention that is substituted by other variants. These results suggest that for iPS cell formation, Sox2, Klf4, and c-Myc are not critically essential. Utilizing Oct3/4 individually in an experiment produced iPS cells from the adult mouse neural stem cells. In that Oct3/4 were expressed in the neural stem cells and generated three iPSC clones, including two clones developing adult chimeric mice, but only a steep contribution from iPS cells determined by the color of the coat. To evaluate if iPS cells can be produced from several mouse cells and human cells utilizing Oct3/4 individually, more research is needed (Takahashi and Yamanaka 2006).

Numerous studies discussing in vitro disease modeling and cell therapy approaches in experimental animal models have generated the presumption that iPSCs will deliver a similar therapeutic efficacy as hESCs and the effective, replicable technique of generating iPSCs. As such, patients with many monoallelic and complicated genetic abnormalities also have developed iPS cell lines (Takahashi and Yamanaka 2006). The iPS cells are quite related to ES cells. In morphology, proliferation, expression of certain ES cell markers, and the development of teratomas, the first-generation iPS cells were identical to ES cells. Fortunately, they noticed that global gene expression and histone methylation varied from their ES cell controls when Takahashi and Yamanaka first identified their iPS cell lines. Methylation and demethylation epigenetic modifications operate to open and close the DNA to transcribe genes and may have a major effect on the cells' ability. Nevertheless, these iPS cells had a distinct form of general gene expression than ES cells and were unable to generate chimeric adult mice. In 2007, germline transmission was accomplished using mouse iPS cells and human fibroblasts developed iPS cells (Takahashi and Yamanaka 2006; Wu and Hochedlinger 2011; Okita et al. 2007).

In animal models of spinal cord injury, retinal disorders, and Parkinson's disease, the therapeutic consequences of human ES cell-mediated progeny were documented. The first clinical trial to target patients of injury in the spinal cord through human ES cells was approved in January 2009 by the Food and Drug Administration (FDA) (Wu and Hochedlinger 2011). Patients with amyotrophic lateral sclerosis (ALS) neurodegenerative disease (Dimos et al. 2008) and patients with other disorders, along with juvenile type 1 diabetes mellitus, Parkinson's disease (PD) (Park et al. 2008), and spinal muscular atrophy (SMA), were able to produce iPS cells (Ebert et al. 2009).

The iPS cell innovation could effectively resolve two significant hurdles associated with human ES cells: post-transplantation immune rejection and ethical issues concerning human embryo. Even then, there are many more challenges, several related to ES cells, as well as some are specific. Teratoma formation is the

first common barrier (Wu and Hochedlinger 2011). Although a limited number of undifferentiated cells can develop teratoma, which is why it is important to induce hES cells or iPSC further into the appropriate form of cells, keeping minimal undifferentiated cells remaining. The existence of transgenes on iPS cells is another significant question. Mostly iPS cells are formed by the transduction of somatic cells incorporated in the host cell genome with retroviruses or lentiviruses that bear transgenes. Transgenes in iPS cells are generally suppressed, but their revival especially the c-Myc transgenes can result in tumorigenesis (Okita et al. 2007). Even if there is a possibility of iPS cells lacking c-Myc can be produced, reactivation of the remaining three factors for reprogramming can induce tumors as well. In addition, persistent transgenic induction could inhibit the differentiation of iPS cells, leading to a greater risk for teratomas being transplanted into patients. Particular obstacles also have to resolve prior to iPS cells being used in a facility mainly concerned with induced somatic cell reprogramming. Failure to reprogram somatic cells to iPS cells could lead to the worsening of iPS cells' differentiation into the type of cell needed.

Several groups have developed either retroviruses or lentiviruses for the mouse or human iPS cells. Developed iPS cells have several viral genome integration sites. During iPS cell development, the proviruses are suppressed and endogenous genes are triggered that express all four factors. The usage of retroviruses or lentiviruses poses protection concerns for iPS cells. In several endogenous genes, viral integration can proceed to gene activation (Wu and Hochedlinger 2011). After reprogramming, the activity of exogenous transgenes is suppressed, which is vital for sustaining pluripotency. These genome-integrating viral vectors may lead to insertional mutations that may affect the possibility of differentiation, or even cause tumorigenesis by c-Myc oncogene reactivation (Okita et al. 2007). Thus, strategies for re-programming methods that use viral vectors are considered too dangerous for therapeutic strategies. Numerous approaches to maintain trans-gene-free and integration-free iPS cells have recently been investigated by successfully employing the number of non-integral vectors for iPS cells, including viral vectors and non-viral vectors. The development of iPS cells from the mouse (Stadtfeld et al. 2008) and humans (Zhou and Freed 2009) was focused on the replication of incompetent adenoviral vectors. As a part of their life cycle, adenoviruses cannot be incorporated into the host genome. In fact, the adenovirus vector integration in the cell clones of the iPS cell was not observed while measured. Along with repeated adenoviral infection, the vectors permit for the transient expression, but the efficacy of reprogramming was much poorer than that for retroviral transmission of exogenous genes without integration into a host genome. One potential reason for poor efficacy is that the reproduction-deficient adenoviral vectors used during the research sustain the expression of exogenous genes for about 3-8 days. In mouse fibroblasts, it has been demonstrated that iPS cell generation requires the transgenic expression for at least 8 days, which implies a sufficiently greater level of adenoviral reprogramming factors could be hard to maintain. In order to generate transgene-free iPS cells without incorporation, multiple groups of vectors have been used as viral substitutes, and expression plasmids were among the first to be investigated.

Prolonged transfection into the mouse embryonic fibroblasts of plasmids comprising the four transcriptional factors contributed in unintegrated iPS cells, but with significantly poorer efficacy than viral vectors (Dimos et al. 2008). Utilizing a variety of distinct gene transfer techniques, such as retroviral, lentiviral, and adenoviral vectors and nonviral plasmids, iPS cells have been developed, and recently via direct delivery of recombinant protein. By usage of genome-integrating viral vectors as with retroviruses and lentiviruses, like even a single insertional mutation, leads to iPS cells that would be inadequate for therapeutic use (Carey et al. 2009; Sommer et al. 2009).

Human somatic cells can indeed be reprogrammed by the use of a mini-circle DNA vector to become induced pluripotent stem cells (iPS cells). Minicircle expression vectors have better transfection efficiencies and relatively longer transgene expression compared to plasmids leading to lower suppressing for exogenous genes. Nucleofection incorporated a single minicircle vector carrying four reprogramming factors into the human ES cells, and the minicircle plasmid backbone was further excised and degraded by using an intramolecular recombination system based on PhiC31 (Jia et al. 2010). The minicircle vectors are modified in some applications to eliminate expression-silencing bacterial sequences, where the vectors contain a unidirectional site-specific recombination product sequence in response to an expression cassette in several embodiments.

While introducing pSESAME, an expression vector that promotes the production of the transducible proteins, further effort to attain pluripotency enhances the use of the transducible proteins. The two major factors of pluripotency in embryonic stem cells, OCT4 and SOX2, were genetically merged with a transcription protein transduction transactivator domain facilitating penetration of cells (Gump and Dowdy 2007). This method offers an efficient technique beyond genetic manipulation for the modulation of stem cell characteristics.

The generation of iPS cells using chemicals or small molecules is often prevented viral integration. Chemicals that can substitute some reprogramming factors during iPS cell development have already been reported by some researchers. Given the key functions of Oct3/4, iPS cells may be produced by chemicals that strongly trigger the endogenous Oct3/4 gene. While iPS cells do not display transgene integration, various genetic modifications such as small plasmid fragment integration or chemically induced mutations may be present. In order to detect these genetic changes, the entire genome of iPS cell clones will need to be sequenced using next-generation sequencing technologies. The sequence of transcriptional and epigenetic changes introduced by the four chief transcription regulators can be recapitulated by small molecules and soluble factors and are notably impressive providing their efficiency of use and the absence of irreversible genome alteration. For this case, valproic acid improves reprogramming effectiveness by approximately 8-fold with that of the four transcriptional factors in mouse fibroblasts; BIX01294 boosts reprogramming efficacies of OCT4, KLF4-infected neural progenitor cells and facilitates reprogramming of the mouse neural progenitor cells in the lack of OCT4, however, with extremely poor capacity and the existence of the other three. It is unclear at present whether the sequence of transcriptional and epigenetic events resulting from

ectopic expression of the four core regulators can be recapitulated by small molecules individually (Huangfu et al. 2008; Shi et al. 2008). Alternative approaches to prevent the incorporation of genetic alterations involve the delivery directly into cells of reprogramming proteins or mRNA, instead of their expression from DNA. The carboxy termini of 4 reprogramming factors have been incorporated into a poly-arginine protein transduction domain to produce recombinant proteins that will penetrate and pass the plasma membrane of somatic cells. iPS cells were generated following repeated protein transductions with valproic acid (VPA), a histone deacetylase inhibitor that can effectively boost reprogramming efficiency (Zhou et al. 2009).

By utilization of synthetic RNAs for reprogramming with remarkably strong reprogramming efficiencies has been documented (Warren et al. 2010). Through the use of in vitro transcription, mRNAs like 3′ and 5′ untranslated regions were synthesized. A high, dose-dependent cytotoxicity was discovered by RNA transfection with a cationic vehicle into MEF and human somatic cells. To refine the synthetic mRNA strategy and boost cell viability, improved ribonucleotides and phosphatase treatment were employed in conjunction with media supplemented with the interferon inhibitor B18R.

The most powerful genome editing research tools established to correct or incorporate disease-relevant mutations in iPSCs that could perform a central function in the analysis and application of pluripotency technology are site-specific nucleases (SSNs) (Urnov et al. 2005). It was a long process and expensive for the early zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) sites for genome editing in stem cells. Consequently, their implementation as research techniques has grown relatively slow. Although the capacity of genome editing was illustrated by comprehensive work with ZFNs and TALENs and the universal influence of these SSN technologies was demonstrated. When combined with an optimized single guide RNA that contains homology to a genetic site of concern, CRISPR type 2 systems, a single protein, Cas9, could act as an SSN (Jinek et al. 2012). The sgRNA replaces the normal Cas9-associated bacterial RNAs that typically give the bacterial pathogen DNA aim specificity and guides Cas9 to cause a blunt double-strand break in target DNA with complementarity in the sgRNA to a 20-nucleotide-long sequence. Cas9-mediated genome editing has established the tool of preference for producing SSNs and genetically modifying iPSCs in just a few years (Marraffini 2015).

In recent years, the rise in multiple iPSC development techniques has increased the possibility of using iPSCs not merely as a research platform, but also has become an appealing complementary therapeutic strategy. Combined with the prospect of producing patient-specific pluripotent stem cells, the emergence of multiple genome editing techniques is changing our perception of the molecular mechanisms causing genetic diseases and cancers (Fredriksson et al. 2014). For regenerative medicine, the revolutionary success of induced pluripotency holds great promise. Patient-specific iPS cells can offer valuable drug discovery opportunities and provide unparalleled perspectives into disease processes and may be utilized for cell and tissue replacement therapies in the foreseeable future.

Neurodegeneration is devastating and ultimately fatal types of disorder characterized by the progressive and irreversible loss of neurons from specific regions of the brain or peripheral nervous system lose function over time and ultimately die. Neurons are the building blocks of the nervous system which consist of the spinal cord and brain. Neurons normally are so specialized that they don't replicate or replace themselves which means when they become damaged or die they cannot be replaced by the body. So the recovery of the damaged cells is much more difficult. According to UN General Assembly report of December 2017, neurological diseases are the leading cause of disability and the second leading reason of death globally (Ahn and Jun 2007). 1 billion people suffered from neurological disorders worldwide over which 6.8 million die every year. Examples of neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease. Alzheimer's disease and Parkinson's disease are the most common neurodegenerative diseases. Neurodegenerative diseases significantly affect millions of people worldwide. These diseases are incurable and debilitating conditions that result in progressive degeneration and/or death of nerve cells. This causes problems by progressive loss of cognition and disruption of basic functions with movement (called ataxias), or mental functioning (called dementias). Dementias are responsible for the greatest burden of disease with Alzheimer's representing approximately 60–70% of cases. Although treatments may help relieve some of the physical or mental symptoms associated with neurodegenerative diseases, there is currently no way to slow disease progression and no known cures. The underlying pathways are not yet entirely elucidated, however, and there are no biomarkers for the early detection or successful therapies yet identified for the root causes of these diseases. In addition to developing novel drugs and therapies, a deeper understanding of these pathways is required for the development of potential cures that reverse tissue loss within the brain. Direct analysis of alive individual neuronal cells is fundamentally restricted due to the inaccessibility of the neural network of the human brain. Alternatives for the assessment of the disease growth, various approaches have been introduced, including the post-mortem brain tissue research was performed, which provided the useful and detailed insight into the end-stage disease pathology. However, this method is incredibly restrictive since it is not possible to analyze patient neurons previous to the degeneration and evaluation of biological variations during the early stages of the disease. While transgenic and knockout models of neurodegenerative diseases are commonly used and have provided valuable insights into certain molecular mechanisms of disease progression, the models don't provide an opportunity to research neurodegeneration mechanisms in at-risk human neurons. So replicating human pathogenesis with this approach is often difficult or even impossible (Thomson et al. 1998). The field is troubled by the scarcity of models specific to the human disease for designing new medications to treat these diseases. Researchers have been involved in stem cells for the past decade and the possibility of using them to explore disease pathogenesis and facilitate the development of novel therapies (Takahashi and Yamanaka 2006; Wu and Hochedlinger 2011; Okita et al. 2007). The use of patient-derived disease-

specific iPSCs allows the development of neurons that comprises the individual patient's genetic information.

Induced neuron cells can be derived by transduction of fibroblast with neural transcription factors such as NeuroD1, Ascl1, Myt1l, and Brn2 (Huangfu et al. 2008). The lack of effective therapies gives the researchers a huge burden of providing curative therapies for various neurodegenerative disorders with limited side effects. The iPSC technology has provided researchers with a unique tool to approach the goal of disease-specific stem cell treatment of degenerative disorders with autologous cells. Brain disorders are specifically striking for stem cell uses; as pluripotent stem cells seem to have an inherent proclivity to differentiate into neuronal lineages. Moreover, many brain diseases have genetic defects and are therefore agreeable over the use of advanced technologies of genome-editing. Crucial for the potential of modeling neurodegenerative diseases is the ability to produce well-defined neuronal populations from pluripotent cells. Although ample progress has been achieved, existing procedures are still often complicated, affluent, and difficult and harvest moderately consistent populations of neurons. However, it has been proven conceivable to differentiate human pluripotent stem cells into cells with the prototype resembling dopaminergic neurons, glutamatergic neurons, GABAergic neurons, motor neurons, and medium spiny neurons of the striatum (Shi et al. 2008; Zhou et al. 2009; Warren et al. 2010; Urnov et al. 2005; Jinek et al. 2012; Marraffini 2015; Fredriksson et al. 2014). Interestingly, iPSC-specific diseases have also been developed from patients with neurodegenerative disorders accomplishing new testing methods for the investigation of fundamental processes and evaluation of therapies. Human iPSCs have been generated from patients of neurodegenerative disorders, like AD, PD, and ALS have been effectively differentiated in vitro into disease-specific cellular populations, comprising motor neurons (MNs), dopaminergic neurons, and oligodendrocytes (Narendra et al. 2010). The use of iPSCs has therefore spread to many disease research fields. IPSCs derived from patient samples can be practiced to build patient-specific disease modeling to elucidate specific pathways and to test novel therapeutic approaches. The potential to model neurodegenerative diseases is important for the ability to develop established populations of neurons from pluripotent cells.

Stem cell technology is a promising evolving area that endeavors the research of cell scientists, as well as gives opportunities for therapeutic intervention for the assortment of dangerous and harmful diseases. The risk of these diseases on society reinforces the requirement for greater emphasis on pursuing stem cell treatment which may provide advanced innovations for reconstructive therapies. For analyzing disease pathogenesis and for establishing innovative therapeutics, exact tractable disease models are needed. And for this regenerative medicine purpose, the cultivation of stem cells is proving to be a valuable asset. Since stem cells are the blank/unspecialized pluripotent progenitor cells generated from a developing blastocyst's inner cell mass which have the capability of self-renewable as well as have the potential to differentiate into any specialized cells of an organism with the potential to give rise to all three germ layers, they are ideally beneficial for both to produce these disease models and to obtain the enormous masses of cells needed for cellular

therapy and transplantation therapies (Frank 2009). There are different types of stem cells on the basis of their source – embryonic stem cells, tissue-specific stem cells, adult stem cells, and induced pluripotent stem cells. On the premise of the extent to which stem cells can differentiate into distinctive cell types, there four main classifications are totipotent, pluripotent, multipotent, or unipotent stem cells. The embryonic stem cells are generated from the inner cell mass of the blastocyst. These cells form clusters called germ layers during the embryogenesis: endoderm, mesoderm, and ectoderm, each of which gradually inevitably leads to differentiated cells and tissues of the fetus, and subsequently along, of the adult organism (Fox et al. 2011). They get to be multipotent stem cells once hESCs divide into one of the germ layers, the potency of which is limited to only the germ layer cells. After that, as undifferentiated cells, pluripotent stem cells occur all over the body, and their main capabilities are proliferation under certain physiological conditions through the development of the subsequent generation of stem cells and transformed into specialized cells. All stem cells may demonstrate beneficial for scientific and restorative research, but the benefits and restrictions of the distinctive form are also present (Wooffitt 2011). Any stem cell treatment helps to restore a damaged tissue that does not regenerate in itself. Continuous stem-cell therapy study brings promise to patients who typically do not undergo medication to cure their illness, but rather to relieve the effects of their chronic disease. The spectrum of the possible stem cell-based therapeutic approaches for stem cell research has risen significantly in recent years due to the advancements in stem-cell technology, stem cell-based treatments have been conducted as a clinical norm for treating some diseases such as hematopoietic stem cell transplants for leukemia and epithelial stem cell-based medication for burns and corneal disorders (Imarisio et al. 2008). Medical experts expect that cancer, type 1 diabetes mellitus, Parkinson's disease, Huntington's disease, celiac disease, heart failure, muscle damage and neurological disorders, and several others will soon be treatable through adult and embryonic stem cells. Stem cells are generated and transformed into specialized cell types with properties correlated with cells in different tissues, like muscles or nerves by cell culture. Profoundly adult stem cells from a myriad of ways, including umbilical cord blood and bone marrow, are extensively utilized in treatments. In October 2006, the very first artificial liver cells were created by using umbilical cord blood stem cells (Jankovic and Poewe 2012; Connolly and Lang 2014).

In 2008, researchers from Regenerative Sciences reported the very first published study of effective cartilage regeneration in the human knee using autologous adult mesenchymal stem cells (Fox et al. 2011). There were reports in 2008 of embryonic stem cells harvested from a single human hair (Radhakrishnan and Goyal 2018). Australian researchers (2009) have figured out a way of optimizing mouse muscle stem cell chemotherapy (Kondo et al. 2017). In Kim et al. (2009) it was revealed that they had developed a way to modify skin cells to produce unique "induced pluripotent stem cells" (iPS) for patients, implying that it was the absolute stem cells cure (Xu et al. 2013). For the very first time, human embryonic stem cells have been cultivated without using animal substances under chemically regulated conditions, which is vital for potential therapeutic use in 2010 (Kumar Thakur et al. 2018).

Diabetes affects millions of people throughout the world and is triggered by impaired conditions of Insulin metabolism. Normally, the cellular structures called the islets of Langerhans in the pancreas contain and secrete insulin. Recently, insulin-producing cells have been produced from mouse stem cells. Furthermore, to establish structures that precisely mimic typical pancreatic islets and release insulin, the cells self-assemble (Ahn and Jun 2007). In order to include a stem cell-mediated treatment to cure diabetes to eliminate the persistent need for insulin injections, the scientific investigation will need to explore ways to improve standards for insulin development.

Disease modeling is an effective consideration for understanding the molecular pathways that guide pathophysiology as well as to empower innovative therapeutics to be developed. As primary patient cells are accessible only in terribly restricted quantities, these models generally use transgenic animals or transformed cell lines. As essential understanding patient cells were accessible in exceptionally little amounts, models usually utilize transgenic genetically manipulated animals or transformed cell lines. The significant benefit of animal models is that they can be utilized to investigate disease in vivo, but it is tedious for primary animal cells to separate in massive quantities for applications because high-throughput screening is required. Transformed cell lines are easily available and can be specifically modified to reveal a disease-causing gene of interest. As in any case, the physiological conditions of animal models and transformed cell lines however vary from those of patient cells, significantly explaining why numerous medications in clinical trials do not function successfully in patients. In addition, human iPSCs derived from the somatic cells of patients can provide a suitable supply of cells for cell transplantation therapy, which can effectively avoid immunological rejection and the ethical problems faced by the usage of ESCs (Han et al. 2012; Bilkei-Gorzo 2014). Most significantly, human iPSCs generated from the somatic cells of patients have a patient-specific pathogenic history, so they can therefore present a potential pathway for disease modeling, integrating the gap between animal models and clinical research. To develop prototypes of Mendelian disorders, genetically advance diseases, and infectious diseases, iPSC-derived cells have been utilized. The increasing advancement of technology for iPSCs encourages the adoption of iPSCs in neurodegenerative disease studies and analysis. More than 50 literatures have been released until 2008, in order to illustrate the modeling of neurodegenerative diseases utilizing iPSCs, developed mainly from familial patients though several from sporadic patients (Han et al. 2012). To discover potential therapeutics, especially novel drug targets, and to assess drug efficacy, iPSC-based models are being used. The scientific investigation might utilize these models to diagnose and screen patients into groups that are treatment responsive or not.

5.1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder where the loss of cortical neurons and hippocampal leads to the weakening of memory and

cognitive ability. Loss of neurons is not uniform but varies dramatically in different functional regions (Michaelson 2014). Over 5.4 million people are living with AD in the United States (Ahn and Jun 2007). In order to explore the pathological mechanisms of AD, many scientific hypotheses have been presented, such as the neuroinflammatory hypothesis (Serretti et al. 2007), the amyloid-cascade hypothesis (Praticò 2008), the mitochondrial cascade hypothesis (Maccioni et al. 2010), the tau hypothesis (Ahn and Jun 2007), and the oxidative stress hypothesis (Ricciarelli and Fedele 2017). It is characterized by plaques comprised of a molecule referred to as amyloid that results in the production of tangled twisted fibers made up of protein tau. These plaques are deposits between brain cells. This triggers the loss of brain functions, which is assumed to be the main cause of memory loss and dementia. AD is the most common cause of dementia which causes loss of intellectual abilities, such as thinking, remembering, and reasoning, that is severe enough to interfere with daily functioning. Alzheimer's disease is highly linked with aging and women have a longer life expectancy than men, women account for over two-thirds of the elderly population with this disease. Amnesia is the first symptom of AD occurrence but soon progresses into more severe conditions including confusion, personality changes, anxiousness, irritability, and severe memory and intellectual disturbances. As a result, patients no longer function normally and the average patient dies about 8–12 years after the initial diagnosis. AD symptoms develop because brain cells (neurons) are destroyed. The reason behind this neuron destruction might be the deposition of "plaques" and "tangles" collected around the neurons which are dense and toxic clumps of proteins. Tangles form when fibers inside neurons become twisted, which also force neurons to die. Plaques are made of a protein called β-amyloid (A_β) (37–49 amino acids). β-amyloid comes from a normal protein that neurons need to function normally, called amyloid precursor protein (APP) but, APP is cut by enzymes into abnormal amounts of β amyloid. Neurons can get rid of a small amount of β amyloid, but if it is too much, β amyloid clumps together to form plaques inside of neurons as a result neurons don't function normally and die-off. Secretases are enzymes that "snip" pieces off a longer protein that are embedded in the cell membrane. Among other roles in the cell, secretases act on the amyloid precursor protein (APP) to cleave the protein into three fragments. Sequential cleavage by β-secretase (BACE; beta-site amyloid precursor protein cleaving enzyme) and γ-secretase (Presenilin Complex) produces the amyloid-β peptide fragment that aggregates into clumps in the brains of Alzheimer's disease patients. If α-secretase acts on APP first instead of BACE, no amyloid-β is formed. The symptoms of Alzheimer's disease worsen over time, although the rate at which the disease progresses varies. On average, a person with Alzheimer's lives 4–8 years after diagnosis but can live as long as 20 years, depending on other factors. Complications of the decline in brain functions are what lead to death. In AD, mutations in the genes coding for the amyloid precursor protein (APP) and proteins known as the presenilins, which may be involved in APP processing, lead to inherited forms of the disease. The synthesis of the integral membrane protein APP (amyloid precursor protein) is the key to the development of this disease. To produce sAPP and a C83 carboxy-terminal segment, APP is initially cleaved by

alpha-secretase. The involvement of sAPP is correlated with proper synaptic signaling, leading to synaptic plasticity, memory and learning, emotional activity, and survival of the neurons. In the disease condition in order to trigger an extracellular fragment referred to as A40-42, APP is sequentially cleaved by β-secretase and γ-secretase. Aggregation of this segment leads to oligomerization of A40-42 and neurotoxicity inflicting plaque generation leading to blocked ion channels, calcium homeostasis destruction, mitochondrial oxidative stress, impaired energy metabolism, irregular regulation of glucose, and finally neuronal cell death. Reactive oxygen species produced in the cell even stimulate CDK5 as well as alternative kinases like GSK3, PKC, PKA, and Erk2. This progresses to Tau hyper phosphorylation, leading to the dissociation of Tau from the microtubule, progressing to the destabilization of the microtubule and oligomerization of the Tau protein within the cell. Due to Tau oligomerization, neurofibrillary tangles form and contribute to neuron apoptosis. Experimental novel anti-AD therapies, though human clinical trials aimed at Aβ, were unsuccessful (Ebert et al. 2009). To date, around at 3 responsible genes and 22 risk genes, along with amyloid precursor protein, presenilin-1/2 (PSEN1/2) (Ricciarelli and Fedele 2017), apolipoprotein E (APOE) (Van Eldik et al. 2016), FERMT2, SLC24H4-RIN3, BIN1, ZCWPW1, CD33, HLA-DRB5-DBR1, CELF1, MEF2C, CR1, NME8, EPHA1, CD2AP, INPP5D, MS4A, CLU, DSG2, PICALM, SORL1, ABCA7, PTK2B, and CASS4 (Feigin et al. 2019) have been recognized to be associated in AD pathogenesis. Through over-expressing one or even more mutated genes, the discovery of AD responsible genes led to the development of more than 150 transgenic AD mouse models, which significantly enhanced the knowledge of AD pathogenesis and facilitated the discoveries of potential targeted therapies and AD treatment approaches but neither of these transgenic models, though, may represent all of AD's infective and clinical characteristics. Contrary variations and levels of genetic mutations have resulted in a wide range of AD phenotypes (Bellin et al. 2012; Hargus et al. 2014). Focused on this, transgenic animal models do not completely recapitulate the development of human AD at the existing level. Thus, to promote basic AD studies to evaluate more effective therapeutic methods for AD treatment, more reflective models are required. Despite the causes of AD, all scientific effort and intense research were unclear and any medication to prevent progression, to avoid AD is also lacking (Carey et al. 2009; Sommer et al. 2009). Varieties of medications and therapeutic methods are being tested to delay or avoid neuronal failure and cognitive impairment of AD (Lumelsky et al. 2001). But only five therapeutic agents, which include cholinesterase inhibitors tacrine, donepezil, galantamine, rivastigmine, and N-methyl-D-aspartate (NMDA) receptor antagonist memantine, have been authorized for pharmaceutical AD therapy by the Food and Drug Administration (Kumar 2019). Even so, many of these pharmacological targeted therapies currently listed only alleviate symptoms without altering AD's main pathological features. In addition, the efficacy of such medications affects people differently, as shown by modest efficacy across 20% of cases and side effects, sensitivity, and non-compliance in even more than 60% of patients administered. Induced pluripotent stem cells (iPSCs) technology can offer an attractive approach to AD research to resolve these

obstacles. The recent studies of an AD disease model with iPSCs enable accessibility to cell types that were formerly insufficient and this induces opportunities in the advancement of effective therapies (Ebert et al. 2009). In order to investigate disease progression linked with both inherited monogenetic mutations and sporadic AD, iPSCs have been extensively adopted. This can be achieved by utilizing EB production and neural progenitor cells (NPC) induction strategies in the involvement of some factors, such as N2/B27, progesterone, NGF/BDNF, heparin, dorsomorphin, FGF2/EGF2, cAMP, IGF1, insulin, and SB431542 (Kandasamy et al. 2010; Kabashi et al. 2009; Juopperi et al. 2012; Imaizumi 2012; Hsiao et al. 2014). The neuronal marker characterization can be assessed by expression of TUJI, TH, FOXG1, VACHT/Nkx2.0, CHAT/VACHT/Nkx2.1, VGLUT1/2, CTIP/TBR1, GAD2/1, SATB2, and MAP2 (Hick et al. 2013; Hermel et al. 2004; Hargus et al. 2014).

Potential clinical growth AD iPSC models have been developed from the individual with mutations in gene encoding APP and presenilin, including deletion mutation of E693 and APP duplication of genes exhibiting that they mimic the APP synthesis pathway as well as giving an innovative framework to study the pathogenesis of AD (Kandasamy et al. 2010; Haeusler et al. 2014). Neurons from a case of E693 deletion mutation showed apparent early onset of AD symptoms but lack of Aβ deposition (Fang et al. 2015). They found intracellular Aβ accumulation of oligomers led to the endoplasmic reticulum and oxidative stress (Kandasamy et al. 2010). Cells of patients with a duplication of the APP revealed elevated A-beta peptide and moreover raised phospho-tau and elevated active beta GSK3. These changes were also observed in the endosomal compartment. Several of those modifications could be restored with beta-secretase inhibitors treatment but not inhibitors of gamma-secretase. In addition, the Aβ accumulated oligomers were not immune to proteolytic, and subsequent docosahexaenoic acid (DHA) treatment has remedied the stress reaction in neural AD cells. Such observations can illustrate the specific therapeutic consequences achieved with the usage of DHA therapy and recommend that DHA may indeed be beneficial for a particular group of patients (Kandasamy et al. 2010). The effect of presenilin (PS) mutations observed by models generated by fibroblast of the patients both from PS1 and PS2 results in human iPSCs-derived neurons displaying increased concentration of Aβ 1–42 that reacted to treatment with γ-secretase inhibitors (Egawa et al. 2012; DeJesus-Hernandez et al. 2011). β-secretase inhibitors have been examined in iPSCs, neurons isolated from fAD patients triggered by APP gene duplication and sAD. Compared with non-demented checks, APPDp-patient neurons showed a considerably higher rate of the pathological marker Aβ 1−40, phosphotau (Thr 231), and the active kinase-3β glycogen synthase (aGSK-3β). Treatment of the purified neurons with β-secretase inhibitors induced a decrease significantly in aGSK-3β and phospho-tau (Thr231) levels indicate a strong APP proteolytic processing relation, but not Aβ in activation of the GSK-3β and tau phosphorylation in human neurons (Haeusler et al. 2014). Recent research demonstrated that neurons derived from AD-iPSC increased the intracellular Aβ40, Aβ42, and caspase 1 action, declined phosphorylation of serine 9 in glycogen synthase kinase 3β (GSK3β), impaired neurite outgrowth, and serine 396 in tau protein (Sommer et al. 2009). AD-iPSC cells offer a beneficial

forum for the analysis of possible drug targets as well as subsequent drug growth, as AD-iPSC-derived neurons can illustrate pathological aspects of disease. In AD-iPSC-derived neurons, drug screening has been worked out to look for those agents that can decrease cell death induced by Aβ toxicity. Cyclin-dependent inhibitors of kinase 2 have therefore been established as agents capable of lowering Aβ neurotoxicity robustly (Kim et al. 2009). In addition, the ability to reduce aβ synthesis was also recognized by six therapeutic compounds. A combination of bromocriptine, cromolyn, and topiramate demonstrated powerful anti-Aβ response in AD-iPSC-derived neurons among such compounds (Lim et al. 2016). Thus, iPSCs are an effective alternative therapeutics to traditional medical care, make a positive contributing platform to identify potential drug targets, and could reduce cell death due to Aβ toxicity (Hick et al. 2013).

5.1.2 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disease with a prevalence of at least 2–3% of the population ≥65 years of age in the world in which men are 1.5 times more likely to have this disorder than women unknown, though a role for estrogen has been debated. The mean age of onset is about 60 years but can be seen in 20s and even younger. Globally over 10 million people are living with PD (Ahn and Jun 2007). Parkinson's disease (PD) is an idiopathic disease of the nervous system caused by the confluence of environmental and genetic influences probably involved in producing abnormal protein aggregation within selected groups of neurons evidenced by both motor and non-motor system manifestations, followed by cell dysfunction and then death. It is a chronic progressive neurodegenerative disorder that progresses slowly as small clusters of neurons in the midbrain die. PD appears majorly in older persons but can often occur even in younger patients. Degeneration of the dopamine-secreting neurons (DA neurons) of the substantia nigra in the midbrain which sends fibers to the basal ganglia (sends fibers to the motor cortex) and releases dopamine for movement is the major cause of this disease (Connor 2018; Chiu et al. 2015).

Lewy body is the deposition of abnormal circular structures found in DA neurons with a dense core consisting of a-synuclein protein (presynaptic protein, normally helps in synaptic vesicle function) which is immunoreactive inclusions involving a range of neurofilament proteins along with proteins responsible for proteolysis including ubiquitin responsible for major protein lysis. Mutation on chromosome 4 is one of the other causes of PD as this gene code for the synuclein (SNCA). Lewy bodies are formed by the misfolding of SNCA that causes its aggregation. A mutation on chromosome 6 produces an abnormal Parkin (PRKN) protein. Normal Parkin protein facilitates in trafficking defective/misfolded proteins to proteasomes for destruction (recycling) which helps in ubiquitination whereas defective Parkin allows abnormally high levels of defective proteins to accumulate in dopaminergic neurons as a result it fails to ubiquitinate abnormal proteins consequence killing the cells. Leucine-rich repeat kinase 2 (LRRK2) encodes for the protein dardarin, which

is neuroprotective in nature. Mutation in LRRK2 gene is the greatest known genetic contributor to Parkinson's disease (PD). PTEN induced kinase 1 (PINK1) gene codes for a mitochondrial complex protect cells from stress. Induced mitochondrial dysfunction has been reported to be causal for an autosomal recessive form of PD. Environmental factors that predispose PD include living in a rural environment, exposure to pesticide use and wood preservatives, environmental toxins, cigarette smoking possibly causes mitochondrial dysfunction tends to coordinate with increased possibility of PD. When patients experience physical impairment, the American Academy of Neurology suggests seven types of primary medications considered to alleviate motor symptoms drug treatment in PD patients. They comprise carbidopa/levodopa (Sinemet), dopamine agonists (both ergot and non-ergot types), monoamine oxidase-B (MAO-B) inhibitors, injectable dopamine agonist (apomorphine, or Apokyn), N-methyl-DAspartate receptor inhibitors, and anticholinergics. Levodopa, non-ergot dopamine agonists (pramipexole, or Mirapex; ropinirole, or Requip), and MAO-B inhibitors (selegine, or Eldepryl; rasagiline or Azilect) inhibitors are widely utilized during initial therapy (Jha et al. 2020). Levodopa (L-DOPA), which is a precursor of DA coupled with a peripheral decarboxylase inhibitor, has been considered as the gold standard for the treatment of PD which is generally very effective for the first 2–5 years of treatment but the drug may cause side effects and over time loses effectiveness. Increased level of L-DOPA in the brain causes remaining DA neurons to secrete more DA, alleviates symptoms like hallucinations, psychosis. High levels of L-DOPA produce side effects – acting on DA systems other than nigrostriatal. Actually, amantadine, which is believed to act as an N-methyl-d-aspartate receptor antagonist, is the only current and effective therapeutic medication for l-DOPA-induced dyskinesia (Kalra and Tomar 2014; Thomson et al. 1998; Takahashi and Yamanaka 2006). The enhanced growth over the years of safe and effective cell therapy based on iPSC as an alternative method was demonstrated to enhance the effectiveness of the dopaminergic neurons (Zhou et al. 2009; Chen et al. 2014; Chae et al. 2012). IPSCs unique to patients with idiopathic cases of Parkinson's disease make the generation of DA midbrain neurons having the same genetic makeup as patients and many of them share significant nigral DA neuronal properties throughout the patients of Parkinson's disease (Centeno et al. 2018). For increasing the effectiveness of dopaminergic neuron through iPSC several protocols have been developed for deriving NSCs, cortical neurons, and iPSC dopaminergic neurons in the existence of small molecules and growth factors such as ascorbic acid (AA), noggin, purmorphamine, FGF2, glial-derived neurotrophic factor (GDNF), BDNF, cAMP, Sonic Hedgehog (SHH), SB43152, SB431542, DAPT, Dkk1 blocking ab, FGF8, TGF-β, LDN193189, CHIR99021, and dorsomorphin for the treatment of PD (Hick et al. 2013; Canals et al. 2018; Camnasio et al. 2012; Burkhardt et al. 2013; Bilican et al. 2012; Bidollari et al. 2018; Battaglia et al. 2011; Arai et al. 2006). Human dopaminergic neurons originating from iPSCs can be characterized by the expression of MAP2, SOX2, vGlut1, OTX2, LMX1A, FOXA2, TUJ1, TH, DAT, PAX6, VMAT2, mAP2, EN-1, NURR1, NR1, NeuN, Nestin, GFAP, and GIRK2 genes (An et al. 2014; An et al. 2012; Alami et al. 2014; Zeng et al. 2010; Yue et al. 2012).

In addition, the midbrain, derived from iPSC can transplant DA neurons into adult rodent stratum, where they manifest arborization and integrated mediation consequences as defined by amphetamine reduction and rotational asymmetry, caused by Apo morphine. Though, only a few DA neurons projected into the host striatum at 16 weeks after transplantation (Yagi et al. 2011). While monogenic types of PD contribute only for a minority of cases of Parkinson's disease (Wu et al. 2019), however mutations with these genes the DA neurons degeneration is important in order to understand the mechanism of disease and for the identification of drugs that modify the disease. Mutations in GBA, LRRK2, PARK2, PARK7 (DJ-1), PINK1, α-Synuclein (SNCA), or UCHL1 can be attributed to monogenic forms of PD or enhanced vulnerability to PD, which indicates substantial importance for all of these proteins in disease pathogenesis (Burkhardt et al. 2013). A later study produced cortical neurons from iPSCs reprogrammed from the most prevalent alpha-synuclein patient's mutation, with the A53T. These cells exhibited increased nitric oxide and 3-NT levels as compared with control. Also having evidence of ameliorated ER stress. A small molecule (NAB2) ameliorates certain of those phenotypes (Burkhardt et al. 2013). Mutant in yet another research genetically modified alpha-synuclein, through gene-editing techniques. Also mutant alpha-synuclein cells demonstrated increased nitrosative stress markers. The researchers served a crucial role of the transcriptional MEF2C-PGC1alpha pathway to apoptosis induced by the mitochondrial toxin in A53 T mutant alpha-synuclein neurons, and by good efficiency, the screening detected a small isoxazole molecule as being efficient for activating transcription of MEF2 and protecting neurons from apoptosis (Camnasio et al. 2012). SNCA is the earliest gene linked to autosomal-dominant familial Parkinson's disease, which produces a protein associated with the synaptic vesicle in Lewy bodies that exists in great abundance caused by alpha-synuclein mutations or by gene duplication or triplication through overexpression of normal alpha-synuclein, sync with gain-of-function (Centeno et al. 2018). In recent research, iPSCs had been obtained from a PD patient with triplication of the gene SNCA (Bilican et al. 2012). Relative with normal control DA neurons produced from an unaffected first-generation relative, the amount of α-synuclein generated from this patient was double with the unaffected protein Synuclein (Bidollari et al. 2018). Mutation of gene LRRK2 is the most common mutation linked to PD (Centeno et al. 2018; Yue et al. 2012). In certain populations, mutations in LRRK2 account for up to a quarter of familial PD cases. The most common mutation of L RRK2 is that of G 2019S. iPSCs carrying the p.G2019S mutation (G2019S-iPSCs) in the LRRK2 gene can distinguish into DA neurons and have shown enhanced expression of key genes for oxidative stress response, and α-synuclein protein upregulation (Yue et al. 2012). They were more susceptible to cell toxicity generated by cell stress factors including hydrogen peroxide than control neurons. Phenotypic variations were identified in a subsequent study. The mutant cells between LRRK2 and the control cells, including respiratory changes, though the variations appeared less sturdy (Alami et al. 2014). PINK1 is an encoding gene for mitochondrial kinase, which shields the cells from mitochondrial stress and regulates mitochondrial stress breakdown (Wang et al. 2018). The loss of PINK1 is due to the elevated level of oxidative stress (Tardiff

et al. 2013). Seibler et al. stated that genetic PD-fibroblasts with PINK1 mutations can be reprogrammed and differentiated into dopaminergic (DA) neurons (Chiu et al. 2015). The neurons, upon mitochondrial depolarization, had effected enlistment of lentivirally processed Parkin to mitochondria, raised mitochondrial copy number, and high expression of the significant regulator of mitochondrial biogenesis, PGC-1α (Chiu et al. 2015). Relevantly, these changes were rectified by the lentiviral expression of wild-type PINK1 in neurons derived from PINK1 mutant iPSC (Chiu et al. 2015). Some variations in the metabolic parameter of the PINK1 neurons relative to LRRK2 neurons, however they were observed to be identical in cellular vulnerabilities. Co-enzyme Q10 or rapamycin may help rescue cell vulnerabilities. Interestingly, the mutant cells LRRK2 as well as the PINK1 mutant cells may help rescue LRRK2 kinase inhibitor GW5074. So the models of the PD iPSC propose a pathogenic process where PINK1 is upstream of LRRK2 and LRRK2 is Alpha-synuclein upstream. A further idea has been determined from a pathogenic process of human PD iPSCs that the Parkin recruitment is hindered in patients mutant derived neurons on PINK1. This will put Parkin in a pathogenic process, downstream of PINK1. iPSC PDs from patients with parkin also displayed evidence of enhanced oxidative stress and increased activation of the associated NRF2 cascade (Tardiff et al. 2013). There have been numerous reports demonstrating transplantation of iPSCs into PD models. An initial research demonstrated neuronal human-originated iPSC cells could be transplanted into the brain of a fetal mouse. The cells moved to multiple brain locations, with glial and neuronal differentiation. There were several instances of better psychological phenotype when iPSCs were triggered to differentiate into dopamine neurons and transplanted into a rat model of PD. The cells were pre-screened to differentiate by fluorescent-activated cell sorting to separate out pluripotent cells, susceptible to tumor formation (Takahashi et al. 2007). Research also demonstrates that differentiated PD iPSCs into dopamine neurons could be transplanted into adult rodent striatum and some of these cells generate projecting axons within the striatum. 6-OHDA lesioned rats transplanted with the neurons had lower apomorphine- and amphetamine-induced rotational asymmetry (Wu et al. 2019). Further research demonstrates that the human protein-based transplantation (developed with no viral or other DNA-based vectors) of iPSCs into rat striatal lesions could retrieve motor disabilities (Sreedharan and Brown 2013). Leading to a shortage of sophistication and neuronal immaturity, which are particularly problematic for modeling adult-onset sporadic diseases, disease modeling with 2D iPSC-originated cultures may not be ideal. Recently established brain organoid techniques will facilitate in construction of complicated 3D midbrain tissue models from iPSCs. Owing to the presence of well-characterized neurons, astroglia, and oligodendrocytes, these midbrain organoids are promising models for phenomenological research and regenerative medicine development for PD. The genetic characteristics of the brain and intestinal organoids were, however, inherited from PD-iPSCs bearing the LRRK2(G2019S) mutation have been changed relative to controls. Parkinson's disease (PD) is a fascinating model for utilization of iPSC technology, as procedures for synthesis dopamine neurons (which are not the only

neurons affected, but which are preferentially vulnerable) are comparatively robust and reproducible.

5.1.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive neurodegenerative disorder that remains fatal by primarily destroying the motor system, and it also often affects neuroanatomical regions involved in cognition and behavior. This rapidly progressive degeneration of upper and lower motor neurons from the motor cortex, brain stem, and spinal cord results in denervation, weakness, and wasting of muscle in the arms, leg, trunk, and bulbar region. Amyotrophic lateral sclerosis (ALS) is a late-onset fatal neurodegenerative disease usually appears between the ages of 40–70 and affects 20% more men than women with a prevalence of about 2 in 100,000 people with an incidence of 1.7 per 100,000, remarking minimal average survival. Four out of 10,000 people in the United States are affected with ALS (Ahn and Jun 2007). Most ALS cases are sporadic with unknown genetic etiology, but 5–10% of the cases are familial ALS. Both sporadic and familial ALS (FALS) are associated with degeneration of cortical and spinal motor neurons or pathways between the brain and spinal cord and the muscles of the body pose a serious disability and inevitably death due to ventilatory failure. Patients mostly live up to 3–5 years. Symptoms include muscle weakness in one or more of the following: hands, arms, legs or the muscles can spread to the mouth-swallow area and then can spread to the tongue. This expansion can cause problems in speech and swallow ability. In severe cases, it can cause breathing problems. The fundamental pathogenesis mechanism of ALS is not completely understood, but the neuropathological hallmark of the disease is defects of RNA processing, the aggregation of the protein, and accumulation of ubiquitylated proteinaceous inclusions in motor neurons. In some cases of ALS, a gene causes a mutation in a protein (called SOD1 Copper/zinc superoxide dismutase) that normally "cleans" up toxic particles inside a cell, and causes accumulation of toxic particles inside motor neurons causing them to malfunction. SOD1 mutations suggested a possible primary role in oxidative stress implicated in the pathogenesis of neurodegenerative diseases, including ALS related to the superoxide dismutase function. Oxidative stress is probably a secondary component of pathogenesis.

The genetic cause of ALS is a hexanucleotide repeat of GGGGCC, either in the promotor or intron 1 of the gene. The normal range of repeats is uncertain but usually considered up to 20 repeats. About 90% ALS cases account for sporadic with unknown mutations, 10% of the ALS cases are of familial type occurred by mutations of one out of 32 known genetic loci (Shimada et al. 2011) like SOD1 (superoxide dismutase 1) (Shaltouki et al. 2015), transcription factor NF inclusion TDP-43(TAR DNA binding protein-43) (Seibler et al. 2011; Pu et al. 2012), C9ORF72 (Pang et al. 2011; Nguyen et al. 2011) and fused in sarcoma (FUS) (Muratore et al. 2014; Moore et al. 2015). Repeat expansions in the chromosome 9 open reading frame 72 gene (C9orf72) are one of the most known genetic

mutations of ALS which showed mutation in RNA foci or RNA dysfunction from the repeat expansion could be modified having antisense oligonucleotides and are seen in estimated 40% of patients with family background and estimated 10% of those without (Mattis et al. 2012; Marton and Ioannidis 2019). This one component has been used by iPSCs demonstrating that the repeat expansion originates anomalous structures of both RNA and DNA as well as generates nucleolar stress (Liu and Chun 2011). Impaired DNA repair was suggested to have a role in ALS pathophysiology mechanism following the identification of FUS mutations, although the exact role of DNA repair failure in ALS is not clarified. Mutations in DNA repair protein like NEK1 and C21orf2, both can cause ALS (Liu et al. 2012; Lesage and Brice 2009; Kriks et al. 2011), although the biological pathways associated with their causal role is not confirmed yet. There is no cure for ALS and currently, the only two medications with moderate effects that have Food and Drug Administration authorized for the treatment of ALS: riluzole, a glutamate receptor antagonist whose therapeutic mechanism of action is unclear but has an effect on sodium channels and calcium-activated potassium channels, and, new in 2017, edaravone, a free radical scavenger suppressing free radical. They may reduce the progression of the disease and prolong survival for a few months but it has no lasting effect (Kondo et al. 2013). In recent years ALS-patient-specific iPSCs differentiated and transplanted into patients for direct generation or motor neurons for knowing the pathomechanism of the disease and development of new therapeutic treatment of the disease (Kolagar et al. 2020). Retinoic acid (RA), SB431542, purmorphamine, cyclopamine, LDN193189, CHIR99021, compound E (a notch inhibitor for blocking proliferation of cell) helps in differentiation of efficient iPSCs motor neurons (Israel et al. 2012). In recent studies, iPSCs ALS disease models were developed to investigate the pathogenesis of ALS and to evaluate drug approaches (Hoepken et al. 2007; Hargus et al. 2010; Guarnieri et al. 2018). In cytoplasmic inclusions, TDR43 is found in 95% of mutations that cause ALS and about 4% of familial ALS caused by TDP-43 mutations (Gouras et al. 2015). Two studies have shown phenotypes linked to disease in cells programmed from TDP-43 mutant patients (Hargus et al. 2010; Guarnieri et al. 2018). Each of these observations reported high levels of insoluble detergent in TDP-43 protein, in accordance with earlier aggregate studies among patients with ALS. Both studies have revealed diminished mortality and enhanced susceptibility to cell stressors. These findings are compatible with the TDP-43 theory of mutations act through a genetic gain-of-function and imply reactions may be autonomous in cells. Another study found that the number of Staurosporine sensitivity in iPSCs among TDP-43 patients mutates as well as variations in TDP-43 increased themselves and a Micro-RNA (miR-9), which is a TDP-43 target (Ebert et al. 2013). Abnormal axonal transports were observed in iPSCs from TDP-43 mutant patients (Devine et al. 2011). iPSC-derived MNs drawn from ALS patients carrying TDP-43 mutations form cytosolic aggregates similar to post-mortem aggregates tissue of ALS patients displaying shortened neurites, as witnessed in ALS zebrafish model (Guarnieri et al. 2018). SOD1 mutation of ALS is the most studied mutation of this disease (Dettmer et al. 2015). Approximately 20% of ALS cases induced by hereditary changes are

correlated with the Cu/Zn superoxide dismutase (SOD1) gene. As it has been as of late appeared, iPSCs inferred from ALS patients bearing A4V or D90A transformations and differentiated to motor neurons illustrate SOD1 aggregates in cytoplasm and nucleus but not in mitochondria. SOD1 ALS motor neurons showed neurofilament accumulation in cytoplasm and neurites taken after by neurite degeneration that was uncommon in non-motor neuron ALS cells or wild-type and isogenic regulate motor neurons (Dawson and Dawson 2003).

Presently, more than 30 different mutations have been described in ALS patients out of which MNs originated from M337V-TDP-43-iPSCs were more subject to cellular stress and reported greater susceptibility across a range of in-vitro culture assays (Hargus et al. 2010; Guarnieri et al. 2018). IPSCs have recently been utilized as a single constituent in the analysis that shows the repeat expansion generates anomalous structure of both DNA and RNA structures and generates nucleolar stress (Cooper et al. 2012). A high content chemical screen with endpoint aggregates of TDP-43 in upper motor neurons as well as lower motor neuron-like cells authorized FDA-certified small molecule modulators like Digoxin, proving the probability of iPSC-based patient-derived disease modeling for treatment (Chang et al. 2019). Thus, iPSCs differentiation against motor neurons offers an innovative field for the treatment of ALS and creating modern therapeutics for neurodegenerative disorders.

5.1.4 Huntington's Disease

Huntington's disease or Huntington's chorea is an inherited, a monogenetic, autosomal dominant, and progressive neurological disease caused by a mutation in the huntingtin gene (htt) encodes for a protein called the huntingtin (Chambers et al. 2009; Cavalleri et al. 2018). This leads the expression varied with cytosine, adenine, and guanine (CAG) trinucleotide repeat expansion in exon 1 of the huntingtin gene on HD chromosomes (gives rise to GABAergic neuron, i.e., inhibitory neuron death) (Hick et al. 2013; Byers et al. 2011; Wu and Hochedlinger 2011). Normal htt facilitates the production and transport of brain-derived neurotrophic factor (BDNF), growth, and repair critical for the survival of neurons. Abnormal htt becomes misfolded and forms aggregates in the nucleus. As a result, a substantial loss of medium-sized spiny projection neurons within the striatum and, at later stages of the disease, also by degeneration of neurons within the cerebral cortex in patients is linked with a distinctive neuropathologic phenotype such as incoordination, jerky limb movements chorea and dystonia, cognitive and emotional decline and behavioral difficulties. GABAergic neuronal death removes inhibitory control of motor areas in the cortex (hyperkinetic). The mutant type contributes to both the increase of function and the loss of function which has been investigated by several models includes cells, fibroblasts, C. Elegans, drosophila, rats, mice (more than 10 known), rabbits, sheep, and monkeys (Okita et al. 2007; Lowry and Plath 2008). The unusual neuroleptics are clozapine and olanzapine. They each have an antichoreatic influence. Clozapine involves the regulation of white cells in the blood and is thus less effective, making olanzapine the drug of choice. Weight gain and anti-depressive

symptoms are perhaps the most commonly recorded side effects. Some evidence can be found for recommending quetiapine, zotepine, ziprasidone, and risperidone from limited case studies. Although tetrabenazine, a dopamine-depleting drug, was shown to considerably decrease chorea in a controlled trial (Byers et al. 2012). Depression and sedation are the most severe side-effects. Alpha-tocopherol amantadine, baclofen, cannabidiol, chlordiazepoxide, choline, clonazepam, creatine, deanol, dextromethorphan, fluoxetine, idebenone, ketamine, lamotrigine, levetiracetam, milacemide, minocycline, muscimol, OPC 14117, PUFA, remacemide, and riluzole are a growing amount of drugs without or with quite restricted outcomes. Utilizing antiparkinsonian medications, drug treatment for HD has been attempted, but almost always with very poor outcomes. Dopaminergic medications are, thus, not prescribed in general. To date, no medication is effective with any neuroprotective or disease-delaying effect, following several reports. Diseases modifying drugs are being produced, but not accessible.

A few conventions have been established to produce medium spiny neurons, as the chief cell sort in HD, from iPSCs (Bose and Beal 2019; Bali et al. 2017). Derivation of HD-related neurons like GABA medium spiny neurons (MSN)-like neurons, striatal neurons, and astrocytes under N2/N2B27 media with valpromide, Y27632, bFGF, BDNF, and ciliary neurotrophic factor for the recapitulation of HD-related phenotypes, disease modeling, and mutant huntingtin (mHtt) protein aggregation (Hick et al. 2013; Chae et al. 2012; Baden et al. 2019; Aubry et al. 2008; Arnold et al. 1991; Ambasudhan et al. 2013; Yu 2007). It has appeared that in vitro culture of MSN derived from HD-iPSC has improved glutamate stimulation, caspase activity, and cell death after continued culture under growth factor starvation especially, on the exclusion of BDNF from the medium of cell culture (Fredriksson et al. 2014; Bose and Beal 2019; Yamanaka 2012; Mouhieddine et al. 2014). In addition, these cells displayed a reduction in oxygen utilization rate combined with diminished intracellular ATP concentrations which demonstrated a dysfunction of mitochondrial bioenergetics in patient-derived cells (Fredriksson et al. 2014; Yamanaka 2012). In expansion, neural cells derived from HD-iPS cells were more prone to oxidative stress and excitotoxicity caused by H_2O_2 induced by chronic or pulsative therapy with glutamate. This impact was followed by annoyance Ca^{2+} homeostasis in threatened cells. Eventually, HD-iPS, cell-derived neurons uncovered a significantly higher basal lysosomal activity and an enhanced sensitivity to 3-methyladenine (Zhou and Freed 2009; Bali et al. 2017). Comparative evaluation of protein expression among human embryonic stem cells revealed that healthy donor-derived iPS cells and HD iPS cells uncovered 26 dysfunctional proteins in cells derived from patients. These proteins include antioxidant enzymes SOD1, glutathione peroxidase 1 (Gpx1), and Glutathione transferase (GST) which were substantially downregulated, though oxidative stress-response proteins Prx1, Prx2, and Prx6 were identified in higher amounts in undifferentiated HD-iPS cells (Mouhieddine et al. 2014). Modern studies have documented a great many markers which include TUJ1/MAP2, GABA, Bcl11B, GAD65, GAT1, DARPP32, DLX2, Calbindin, GFAP, and s100b can be recognized for HD-associated neurons originating from iPSC (Liu et al. 2013; Byrne 2014; Al-Shamekh and Goldberg 2014). The rise of

genome-editing advances has revolutionized broad opportunities aimed to establish HD-iPSC pairs utilizing different CAG repeats in the HTT gene of HD-iPSC. Moreover, an analysis of the gene array was accomplished on undifferentiated iPS cells from HD patients, genetically altered by bacterial artificial chromosome (BAC)-mediated homologous recombination to determine isogenic control lines (Yamanaka 2012). This research illustrated that, in comparison to gene-corrected HD-iPS cells, TGF-β cascade molecules, members of the cadherin family, and caspase-related signaling molecules were altogether modified in unfixed HD-iPS cells (Yamanaka 2012). These pathways were too modified in additional HD models and within HD patients' brains (Liu et al. 2013; Byrne 2014; Al-Shamekh and Goldberg 2014).

Entire genome expression evaluation was too conducted on control- and HD-iPS cells which had been differentiated in vitro into neural precursor cells. In this research, 1601 genes were differentially mediated between control and neural precursor cells derived from the patient, and gene ontology evaluation uncovered maladaptive pathways included in proliferation, cell cycle modulation, cell signaling, axonal direction, and cell alignment (Fredriksson et al. 2014). Reliable with post-mortem tissue variations in HD patients and HD transgenic mice, dysregulated neural-derived HD-iPS genes precursor cells like p53, UCHL1, TRK (tyrosine kinase) receptors, EGFR (epidermal growth factor receptor), Syndecan4, SRPX conjointly the HMG box protein 1, which were reported to aggregate within the brains of patients enduring from **AD** (Fredriksson et al. 2014; Byrne 2014). Recently, the expansion of 3D frameworks, incorporating extracellular matrix as well as cell-cell interactions, utilizing spheroids or microfluidic-based innovations, signifies superior interactions among neurons and glia cells for HD modeling based on iPSC (Al-Shamekh and Goldberg 2014). Therefore, these studies demonstrate the potential of including HD iPSCs for the studies of pathogenesis as well as for testing therapeutic research.

5.2 Conclusion

Gathering evidence demonstrates that iPSC researchers have technologically advanced innovative methodologies to analyze the pathophysiology of human diseases and new opportunities for cell-based research and clinical applications as well as regenerative medicines. The perception of this pathogenesis of neurodegenerative disorders is presently underway restricted by challenges in acquiring live neurons from patients and the failure to model the sporadic forms of the disease. Reprogramming adult somatic cells from patients into iPSCs and neurons may overcome these difficulties. Altogether, these iPSC technology findings on neurodegeneration have promising opportunities for biomedical research on human neurodegenerative diseases via sustained propagation and optimal differentiation of patient-derived iPSCs into specific neuronal subtypes that can better recapitulate cell progression toward neurodegeneration in vitro (Fig. 5.1). Such an approach enables the establishment of innovative methods for the modulation of

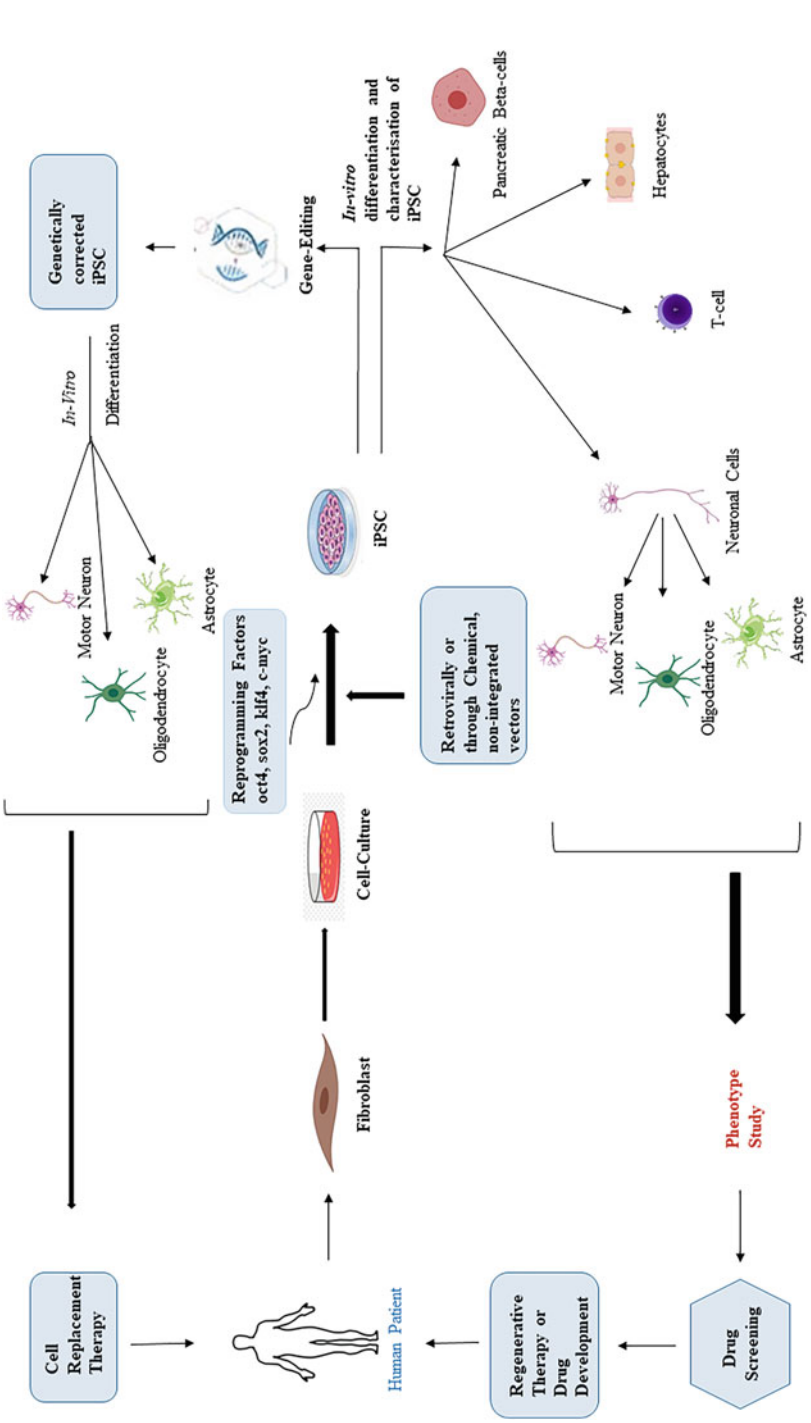

Fig. 5.1 Potential clinical significance of Induced Pluripotent Stem Cells based technology in Drug Discovery and Advancement in cell therapies. Adult somatic cell sources like fibroblasts of patients readily isolated, in-vitro cultured, and are reprogrammed into iPSC via the expression of four transcription

iPSC-derived neurons, and the development of novel disease phenotypes in vitro, with the expectation that will potentially evolve novel clinical therapy. Currently, the field of neural stem cell research inspires the exploration of distinctive properties of neurodegenerative disorders, and in the future, iPSC can be an effective candidate to produce the varieties of human-derived neurons required for regenerative purposes. While many challenges remain to be addressed before iPSC-based technology can be adopted extensively for clinical purposes, the combination of iPSC technology with genome editing and other innovations will undoubtedly speed up the creation of new medicines for human neurodegenerative diseases and can potentially heal these devastating diseases through cell replacement therapy.

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Fig. 5.1 (continued) factors, Oct4, Sox2, Klf4, and c-Myc. iPS cells derived from somatic cells of patients with intractable diseases can be differentiated into the affected cell types like the neural cells type (neurons and glial cells) which are affected in the disease serve as the in vitro disease models for mechanistic studies of disease with the same genetic information as the patient. Neurologic patients' fibroblasts are reprogrammed and further differentiated into neuron subtypes. Neuron phenotypes, such as morphology, connectivity, and synaptic transmission, are analysed in an effort to enhance knowledge of disease pathology, drug screening, and Regenerative Therapy. Moreover, the underlying molecular processes of genetic mutation are also studied through iPSC. By genomic correction of the mutation, iPSCs can be produced. In addition, it is possible to use in vitro differentiated cells for elevated drug screening or validation of novel genetic therapies that can then be translated into therapeutic research

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Imaging of Stem Cell Therapy for Stroke and Beyond

6

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Abstract

Stem cell-based regenerative medicine is an attractive approach for the treatment of neurological diseases such as ischemic stroke. Preclinical studies have shown that transplanted cells can differentiate into neurons and glial cells and improve neurological function. To fully demonstrate and evaluate the potential of stem cell transplantation, the delivery process, long-term tracking, viability, and function of the transplanted cells must be carefully monitored. Imaging techniques that are the most frequently used for stem cell tracking are magnetic resonance, radioactive, and optical imaging. Interventional, real-time magnetic resonance imaging enables precise and safe delivery of stem cells to desired regions in the brain. MRI is also characterized by excellent spatial resolution for long-term cell tracking; however, currently used tracers such as superparamagnetic iron oxide particles do not allow for accessing cell viability. Reporter gene technology allows for studying cell viability; however, so far, reliable reporter genes are only available

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for preclinical studies. In clinical studies, cell tracking was performed with magnetic resonance metal-based tracers and radionuclide imaging. Eventually, numerous techniques were developed to modify the function of transplanted stem cells by external stimuli, e.g., light and ultrasounds. Activation and deactivation of cell functions can be monitored in real time with more advanced imaging techniques, such as functional magnetic resonance imaging.

Keywords

Cell tracking · Imaging · Stroke · Stem cells · Transplantation

6.1 Introduction

Stroke is the second leading cause of death worldwide, with 5.5 million deaths in 2016 (Gorelick 2019). In the same year, 80.1 million people suffered a stroke, of which 13.7 million were new cases (GBD 2016; Stroke Collaborators 2019). Of the 30-day survivors of the first-ever stroke, about half survive 5 years, one-third remain disabled, and one in seven remain in permanent institutional care (Hankey et al. 2002). Treatment of acute stroke and long-term care is costly. Between 2014 and 2015, the direct and indirect costs of stroke management in the United States (US) were $45.5 billion. However, costs are estimated to double by 2035, reaching up to $94.3 billion (Benjamin et al. 2019).

The primary therapeutic goal in acute ischemic stroke (AIS) is to restore cerebral blood flow. Currently, there are two options available: intravenous thrombolysis with tissue plasminogen activator (tPA) and endovascular mechanical thrombectomy (EMT) (Powers et al. 2018). A significant limitation of both approaches is the narrow therapeutic window. The tPA has a 4.5-h therapeutic window, which means only 5–10% of patients are eligible to receive the treatment. The recanalization rate is low, preventing disability in only 55 patients out of 1000 treated without reducing mortality (Wardlaw et al. 2012). EMT has a longer therapeutic window, which is 6 h, even 24 h in some cases, and has a higher recanalization rate of 67.9%. However, this requires immediate access to the interventional suite and a highly skilled endovascular specialist. Such advanced infrastructure and training require tremendous investments. Thus, the impact on population-based health is still limited as of now (Flottmann et al. 2018). Accordingly, despite significant AIS treatment progress, 41% of patients are still with deficits rendering them dependent 6 months after stroke. Therefore, it is warranted to develop therapeutic strategies for patients suffering from permanent neurological deficits.

The central nervous system (CNS) has a severely limited ability to restore damaged tissue. Therefore, stem cell-based regenerative medicine with transplantation of exogenous cells is an attractive strategy for patients suffering from neurological diseases such as stroke, multiple sclerosis, Parkinson's disease (PD), Alzheimer's disease (AZ), etc. Preclinical studies have shown that transplanted cells can differentiate into neurons and glial cells and improve neurological function;

however, functional integration into neuronal circuits is somewhat limited. The exact mechanism of action also remains unknown. Various stem cells are tested in preclinical studies, including mesenchymal, hematopoietic, neural, and adipose-derived progenitor cells, induced pluripotent stem cells, and immortal stem cell lines. Moreover, several clinical trials have demonstrated the benefits of stem cell transplantation in the site of brain damage. Despite significant progress in regenerative medicine, many questions have to be answered regarding the optimal stem cell type, dose, timing, route, and precision of delivery, as well as monitoring the delivery process.

To fully demonstrate and evaluate the potential of stem cell transplantation, the delivery process, long-term tracking, and function of the transplanted cells need to be carefully monitored (Fig. 6.1). Several imaging modalities used in preclinical and clinical studies enable imaging of stem cells. The techniques most frequently used in stem cell imaging are magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and optical imaging. A promising approach combines two or more multimodal imaging techniques and gives more information than a single imaging modality. Each imaging method has advantages and disadvantages regarding image resolution, long-term stem cell tracking, imaging agent sensitivity, image acquisition duration, suitability for clinical use, and cost.

Further progress in cell therapy for stroke may be accelerated with access to the tools facilitating directly analyzing cell therapy benefits, including studies on cell migration, location, division, differentiation, and survival. This chapter focuses on the noninvasive imaging of various aspects of stem cell-based therapy, from monitoring the cell delivery process in real time, early biodistribution, and long-term tracking to function monitoring.

6.2 Real-Time Imaging of Stem Cell Delivery

Precise and safe delivery of stem cells to the CNS is essential for efficient therapy. Any delivery route or procedure needs to be monitored to provide information about the destination of injected cells and their biodistribution. Without detailed knowledge about delivery accuracy and distribution, it isn't easy to fully evaluate and improve the therapeutic procedure. Moreover, also, procedural complications might be monitored in this way. Several approaches are used for cell delivery to the brain: intravenous (IV), intraarterial (IA), intracerebral (IC), intranasal (IN), and intrathecal (ITH). A common and straightforward way of IV application does not allow for precise and targeted delivery; moreover, most injected cells are trapped in peripheral tissue like lungs (Tang et al. 2015). IA and IC routes seem to be the most promising for effective cell delivery to the brain, especially when their precision is validated with real-time (interventional) MRI.

With recent advances in MRI, especially with the development of fast imaging protocols and contrast agents characterized by high sensitivity, it is possible to track stem cells in real time and even predict the biodistribution of injected cells before

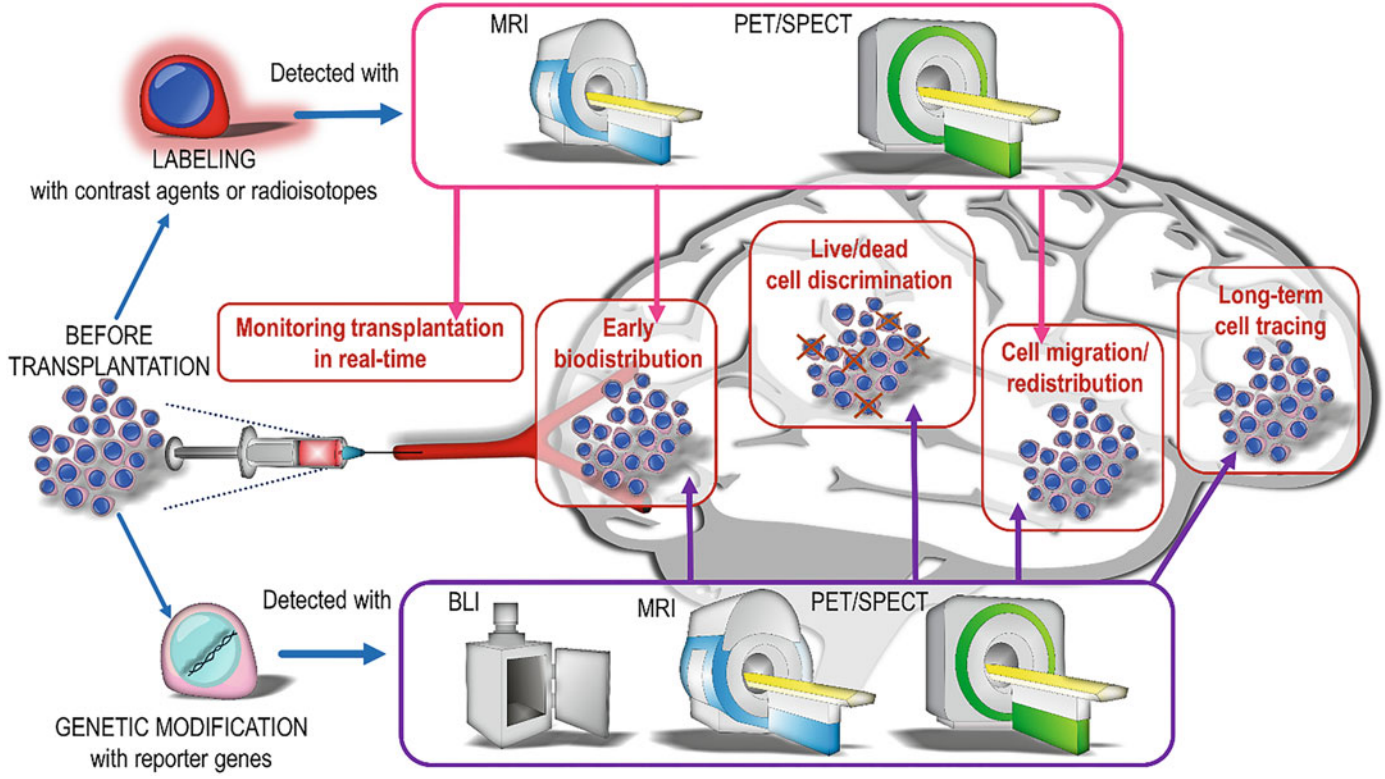

Fig. 6.1 Imaging methods of transplanted cells. Stem cells may be labeled with metal-based contrast agents, radioisotopes, and genetic modifications. Magnetic resonance imaging (MRI) with metal-based tracers such as a superparamagnetic iron oxide (SPIO) allows for real-time monitoring of transplantation procedures. MRI with contrast agents and nuclear imaging with radioisotopes assesses early biodistribution, cell migration, and late distribution. Genetic modifications with reporter genes allow for studying cell viability and long-term cell tracking

their administration. Endovascular techniques provide excellent possibilities for minimally invasive craniectomy-free delivery to the brain, but they require advanced imaging to achieve sufficient precision of neurointervention. After catheter placement in the desired intracranial artery under X-ray guidance, animals are placed in an MRI scanner, and prior to cell injection, a contrast agent is administered. That allows visualization of perfusion territory and tuning of the territory with catheter placement and infusion speed. Once the desired territory is defined, stem cells may be infused. Walczak et al. (2017) showed that using high-speed MRI, based on a gradient echo-echo planar imaging (GE-EPI) pulse sequence, monitoring IA delivery of superparamagnetic iron oxide (SPIO) labeled stem feasible in real time. In a series of experiments with large animals (porcine and canine) and small animal (rats) models of ischemic stroke, they showed that they could predict, adjust, monitor, and define early biodistribution of stem cells. Moreover, they showed that the biodistribution of stem cells is dependent on cell size and infusion speed. Furthermore, IA delivery is an excellent mechanical thrombectomy continuity when patients may be immediately transferred to an MRI scanner for stem cell delivery. However, interventional MRI for stem cell delivery has not been implemented in the clinic yet, even though in 2019 the first MRI-navigated IA delivery to the brain has been recently published, opening a gateway to precise cell delivery to the brain (Zawadzki et al. 2019).

Interventional MRI also enables guiding and monitoring IC delivery. Deep brain electrode implantation, gene therapy, and nanoparticle delivery under MRI have been studied in preclinical and clinical studies; however, IC stem cell delivery sets an entirely new challenge. There is no study about IC stem cell delivery in stroke; however, few studies address this problem in the context of Parkinson's disease. Because the methodological concept for cell transplantation PD and AIS is the same, we will present these studies. IC delivery has to answer questions including precipitation and aggregation of cells in cannulas and infusion lines, flow rate, suspension solution, needle diameter, cell density, backflow along the needle, and needle obstruction. Vermilyea et al. (2017) studied the delivery of induced pluripotent stem cells (iPSC) to nonhuman primates' putamen. Real-time MRI monitoring allowed for better registration of the delivery device, confirmation of trajectory after craniotomy, monitoring of early biodistribution, and identification of issues such as cannula occlusion. Another study showed that the infusion flow rate of stem cells affects dispersion and biodistribution. Malloy et al. (2017) monitored with MRI TurboFLASH sequences, deliverer of neural stem cells (NSC) labeled with SPIO into baboons' brain. They showed that injection 1 ul per minute resulted in cell dispersion with no evidence of cell backflow along the cannula, in contrast to 5 ul per minute.

Interventional MRI also shows early biodistribution of transplanted cells, which may be further followed up with MRI.

6.3 Long-Term Tracking of Cell Survival and Migration

Once the delivery procedure is finished and early biodistribution is established, long-term cell monitoring should be performed to assess transplanted cells' migration, fate, survival, engraftment, and functionality, ultimately providing restoration of tissue function stroke. This paragraph describes methods assessing transplanted stem cells' viability, migration, and biodistribution, beginning with preclinical optical imaging techniques; continuing with clinically translatable modalities like MRI, PET, and SPECT; and finally ending with combining variable techniques in multimodal imaging.

6.3.1 Preclinical Bioluminescence Imaging

The viability and biodistribution of transplanted cells can be investigated by bioluminescence imaging (BLI). The principle of BLI is based on the enzymatic reaction of luciferase. First, transplanted cells need to be modified by introducing a transgene encoding the firefly or Renilla luciferase (Luc) enzyme. After transplantation of Luc-expressing stem cells, luciferase substrate D-luciferin is systemically injected. Luc oxidizes D-luciferin into oxyluciferin, a product that, when decaying, emits photons. Photons emitted by Luc-expressing cells are detected by a specialized charge-coupled device (CCD), allowing for the determination of their organ-level localization. It makes BLI an excellent technique for imaging cells' biodistribution in the scale of the whole body. Moreover, since only viable cells can synthesize enzyme – luciferase – the light emission readout confirms transplanted cells' viability. Pendharkar et al. (2010) used BLI to compare biodistribution of IV and IA delivered NSCs in mice. Immediately after transplantation, they detected a significant difference between transplanted cells' biodistribution. BLI signal from the brain was 12 times higher in IA than IV injections. Early after IA transplantation, 69% of the total signal came from the brain. However, immediately after IV injections, most of the signal was detected in the lungs, and only 27% of the signal was seen in the brain. BLI allows for total body assessment of cell distribution after intravascular therapy and the study of cell location changes in the same animal over time. This technique's simplicity, combined with high specificity and sensitivity and the possibility to quantify signal, makes BLI one of the most utilized imaging tools in preclinical studies. However, BLI is not without drawbacks. First, the low resolution does not provide anatomical details about cells' spatial distribution in the tissue, rather global, organ scale biodistribution. Second, BLI remains a technique suitable mainly for small animal studies due to the high absorption of photons' penetration in soft tissues and bones, making the thickness of the tissue layer the factor strongly limiting readout capacity. Moreover, currently, there is no suitable CCD for humans; thus, BLI is usually used in experiments with small laboratory animals like rats and mice.

6.3.2 Preclinical and Clinical Imaging Techniques

Much more accurate spatial resolution of cell biodistribution can be obtained using other imaging methods currently used in experimental and clinical studies, such as MRI and nuclear imaging: PET and SPECT (Vahidy et al. 2019). MRI is an imaging technique used in the clinic since the 1970s and allows for stem cell tracking in humans. MRI is based on the assessment of the distribution of hydrogen atoms in tissues. Each brain regions, ventricles, white matter, gray matter, and basal ganglia, has different water and macromolecule (lipids and proteins) content, which determines contrast between brain structures. When exposed to the external magnetic field, the nuclei of protons contained in water molecules are excited, which leads to the appearance of their magnetization vector, which is specifically oriented. Switching off the magnetic field reverses magnetization vectors of the proton nuclei to their original state. The MR detection coil measures the degree of proton nuclei magnetization and the time required to restore the magnetization vector original state, known as relaxation time. T1 scans' magnetization vectors of protons' nuclei after excitation are located parallel to the external magnetic field vector. Their decay time, known as T1 relaxation time, is measured in this type of imaging. The additional use of the second alternating magnetic field source leads to a change in the orientation of the magnetization vectors of the hydrogen's nuclei, which are perpendicular to the magnetization vector of the static magnetic field and are called transverse magnetization vectors – their decay, known as T2 relaxation time, is measured in T2 scans. In vivo cell tracking with MRI requires cells' labeling before transplantation with compounds that can change the proton relaxation time, thus increasing the contrast between displaying structures – known as MRI contrast agents.

Stem cells can be labeled using metal-based compounds, genetic-based agents, and some elements such as fluorine-19. Metal-based agents are classified in principle into positive (e.g., gadolinium and manganese chelates)- and negative-like SPIO, based on their effect on relaxation time and signal intensity. Positive tracers decrease the T1 relaxation time, increasing MRI signal intensity (known as a hypertensive signal) in the T1-weighted images. In contrast, negative tracers decrease the T2 relaxation time, resulting in reduced signal (known as hypointensive signal) on T1 and a T2* (a modified version of T2 scans)-weighted images. Since the 1990s, transplanted cells have been labeled and tracked with MRI contrast agents; however, this strategy has gained significant criticism in recent years. The most critical limitation of MRI cell tracking using SPIO and other metal-based tracers is that it cannot discriminate live from dead cells. This is the most significant drawback because it is not known if the image represents living cells. Other limitations are (1) gradual signal loss due to dilution of contrast agent after each cell division; (2) false-positive signal generated by magnetic particles internalized by phagocytic cells in nearby tissue; (3) false-positive hypointense signal caused by air, and blood extravasation, which may result from the delivery procedure; (4) inaccurate quantity of labeled cells; and (5) contrast agents' toxicity (Guzman et al. 2018; Manley and Steinberg 2012). All these limitations cause that, despite excellent anatomical MRI

images, the investigator cannot be sure whether MRI images show living, integrated, and functional cells at the end of the tracking process.

There is a great effort to overcome these limitations with novel imaging techniques such as magnetic particle imaging (MPI), chemical exchange saturation transfer (CEST) MRI, and other MRI contrasts. To distinguish SPIO hypointense signal on T2/T2*-weighted MR images, cells may be concomitantly labeled with opposite contrast agents or selective elements such as fluorine-19. Gadolinium chelates are the most effective paramagnetic agents and are the most popular contrast agents used in the clinic. However, due to its toxicity on cells and tissues, its use in tracking cells is limited. Next to gadolinium, manganese is the second most used "positive" T1 contrast agent. Double labeling of one group of cells, or two, a diverse group of cells with positive and negative tracers may distinguish hypointense T2/T2* regions between transplanted stem cells and artifacts. Gilad et al. (2008) compared SPIO- and MnO-labeled cell injected IC into contralateral hemispheres. SPIO-labeled cells appear as hypointense regions (dark spots), while manganese-labeled cells appear as hyperintense regions (bright spots). The hypertensive area was induced by MnO, and not resulted from edema caused by cell injections, because the control hemisphere injected with unlabeled cells didn't show any contrast. The main advantage of using dual contrast labeling is distinguishing transplanted cells from blood−/hemosiderin-associated hypointense regions; however, it still does not reflect cell viability.

Interestingly, further studies investigating MnO toxicity were performed. Both low and high doses of MnO were toxic; however, cells labeled with lower MnO concentration can recover when the labeling process is terminated. Any cell toxicity was observed in the SPIO group. Another approach for interpreting hypointense T2 signals is cell labeling with selective atoms like fluorine-19 (^{19}F). Fluorine-19 minimizes the signal interpretation ambiguity problem due to the absence of background signal from the tissue. It allows direct detection of labeled cells for unambiguous identification and quantification. However, the sensitivity detection limit of ^{19}F MRI is 200–1000 labeled cells, which is remarkably lower than T2* SPIO signal, which can detect even one cell (Boehm-Sturm et al. 2011; Tennstaedt et al. 2015). Like SPIO, fluorine labeling has no toxic effect on cells (Boehm-Sturm et al. 2011; Tennstaedt et al. 2015). Muhammad et al. (2017) compared MRI imaging of SPIO and ^{19}F-labeled adipose-derived stem cells in skin burns. Due to microhemorrhages in injured tissue, susceptibility artifacts make the SPIO MRI hard to interpret, and the cells were difficult to separate from the background. Such situations occur in any acute brain damage like traumatic brain injury or stroke. In contrast to SPIO, ^{19}F MRI showed excellent detection of transplanted cells within the local tissue, as presented by background-free, "hot spot" signal. Equally important was a good correlation between the disappearance of the BLI and 19F MRI signal, which correlates with cell viability.

Poor fluorine-19 sensitivity and the limited availability of the hardware (dual-tunable coils and interfaces) may be overcome by a novel technique called magnetic particle imaging. MPI is an emerging tomographic technique with the potential of simultaneous high-resolution, high-sensitivity, and real-time imaging. It allows

SPIO tracking and quantifying nanoparticle concentration without tissue background noise. In contrast to MRI, it is not a structural imaging technique but a tracer imaging technique more similar to PET and SPECT. MPI uses the specific magnetic properties of SPIO to cut off its signal from water-generated background signal. The curve of SPIO magnetization created under exposition on magnetic field gradient is not linear. In most frequencies of the applied magnetic field, the SPIO excitation state seems steady and fully saturated (saturation is a state when despite constant magnetic induction, the magnetization of a particle does not increase further). However, under specific, narrow magnetic field frequency, a rapid shift of SPIO magnetization state occurs and stabilized again after a further magnetic field frequency change. This phenomenon is typical for SPIO and does not happen in the case of signals from water. During the gradient magnetic field application, the detection coil in MPI collects signals derived solely from magnetizations highly variable over time. Therefore, detection is limited to SPIO-generated signal during magnetization shifting. It provides an image without tissue-water-generated background signal. When combined with MRI, MPI provides excellent sensitivity with a high-resolution image of nanoparticles (Wu et al. 2019).

Cell survival estimation is a common problem for all imaging techniques using exogenous labeling agents that continue to display contrast when the cells are dying or lose contrast during proliferation. CEST MRI is an alternative or complementary to the mentioned above technique that may potentially be used to track cell viability. CEST enables detection of molecules (e.g., proteins) diluted in water which protons undergo excitation in magnetic field frequency other than water's protons. These excited protons can be exchanged with unexcited water protons in multiple repetitive cycles known as chemical saturation transfer, ultimately influencing the water's MRI signal. Since pH is dependent on protons' level in the tissue, it can also be assessed using CEST. Chan et al. invented nanosensors detecting pH changes related to inflammation and cell death, which may be visualized by CEST MRI (Chan et al. 2013). Another approach, which may be used in CEST, is to design CEST probes using paramagnetic metal complexes similar to those used as T1 agents (McMahon and Gilad 2016). Thus, CEST MRI allows for high-resolution, noninvasive, real-time monitoring of transplanted cells, evaluating their viability. CEST MRI is an excellent alternative for another group of imaging methods based on reporter gene technology described below. MRI is a useful tool that may be used in preclinical as well clinical studies. It provides excellent spatial resolution, is noninvasive, and in contrast to radionuclide imaging, does not expose the patient to the radiation what allows for multiple, repeated studies.

Nuclear medicine (PET and SPECT) is widely used in preclinical and clinical studies due to the abundance of different radiopharmaceuticals, high sensitivity, and good tissue penetration. To enhance imaging quality, both techniques may be complemented with computed tomography (CT) and MRI. The external camera detects gamma radiation produced by radionuclides. The main differences between SPECT and PET are the types of radioisotopes that are used. In SPECT, radioisotopes directly emit gamma radiation. In contrast, for PET, radioactive nuclei first generate a positron, annihilating with the electron from the body, producing two

collinear gamma rays that are detected coincidently. The most commonly used isotopes in SPECT are technetium-99 m, indium-111, and gallium-67, whereas in PET, gallium-68, copper-64, and zirconium-89, as well as for nonmetallic radionuclides, ^{18}F, ^{11}C, ^{13}N, ^{15}O, and ^{124}I, are used. Tracking stem cells with PET and SPECT may be categorized into direct and indirect. Direct tracking is when stem cells are labeled with radiotracer in vitro before transplantation, while indirect tracking is based on the reporter gene/probe system.

Because of high sensitivity, radionuclide-based techniques are suitable for imaging early biodistribution in preclinical and clinical studies. Wang et al. (2020) tracked human neural stem cells' (hNSCs') migration into the brain via nasal administration in mice. Cells were directly labeled with ^{89}zirconium ((89Zr)-hNSCs) and tracked with PET/CT. Brain scans were performed at various time points until 168 h from cell administration. Early biodistribution showed that radioactive compounds were distributed mainly in two locations: the nasal olfactory and the ethmoid. The radioactive uptake in the nasal cavity and olfactory bulb gradually decreased over time. The radioactive uptake of the pituitary and pons first increased and then reduced so that any signal was detected from the brain after 4 h. This study reveals one of the most significant disadvantages of radionuclide-based cell tracking. The period of detection of radioactive isotopes closely depends on the half-life of the radioisotope. The use of zirconium-89 (with a half-life of 78.41 h) allows only early biodistribution to be assessed, so based on PET imaging alone, it is unknown whether the lack of signals was due to ineffective intranasal delivery of hNSC to the brain or complete radioisotope decay. In the clinic, Rosado-de-Castro et al. (2013) used SPECT to compare biodistribution of IA and IV delivered technetium-99 m bone marrow mononuclear cells in 12 stroke patients in the subacute stroke phase. Interestingly, after 2 and 24 h, imaging showed that both groups (IA and IV) have comparative, low cell uptake in the brain. IV group had lower radioactive counts in the liver and spleen and greater counts in the lungs at 2 and 24 h compared with the IA group. Radionuclide imaging is an excellent technique for assessing biodistribution in preclinical and clinical studies; however, the tracking period depends on the half-life of radioisotope used. Technetium-99 m is currently the most used radionuclide due to its 6-h half-life, allowing SPECT imaging up to 24 h. A significant PET/SPECT limitation is that radionuclides cannot cross the intact blood-brain barrier (BBB). Thus, imaging is only possible during the early phase of brain injury when the BBB is compromised. Moreover, it cannot distinguish live from dead cells because radioisotopes remain active even after cell death, so it does not allow for imaging of migration, late biodistribution, and cells' viability. For long-term cell tracking in MRI or PET/SPECT, we should refer to reporter gene technology techniques.

6.3.3 Reporter Genes

For reliable long-term tracking, the imaging tag must be passed on to daughter cells during proliferation and rapidly lost after labeled cells' death. These conditions

could assure long-term observation of transplanted cells' biodistribution with a concurrent assessment of cell viability. These properties are ideally addressed by reporter genes used in MRI and radionuclide imaging. The main advantage of reporter gene-based imaging is that only viable cells can synthesize the protein tag and can be visualized. Furthermore, tag protein expression can be controlled by promoter of another gene, allowing protein synthesis only in predefined conditions (Jurgielewicz et al. 2017).

MRI reporter gene imaging can longitudinally track transplanted cells' viability, through migration, until late biodistribution. MRI reporter genes are categorized into three classes, based on the types of encoded proteins: (1) enzymes (e.g., tyrosinase and beta-galactosidase), (2) receptors of the cells (e.g., transferrin receptor (TfR)), and (3) endogenous reporter genes (e.g., ferritin reporter gene). Reporter genes provide an excellent technique for tracking cells and assessing late biodistribution. Moreover, they overcome the disadvantages of SPIO labeling, e.g., losing signal in time. Huang et al. (2019) compared migration and biodistribution of bone marrow mesenchymal stromal cells (BMSCs) labeled using different approaches in a rat stroke model. In one group, stem cells were labeled with SPIO; in the other group, transduced with a lentivirus containing a shuttle plasmid carrying the ferritin heavy chain 1 (Fth1) gene (Fth1-BMSCs). On day 10, SPIO-labeled cells appeared as a low-signal intensity spot in the T2-weight images around the lateral ventricles, and the signal was then gradually lost.

On the contrary, Fth1-BMSCs on day 10 were seen in the cortex of the right hemisphere. MRI imaging showed cell migration along nerve fibers to the right side of the infarction lesion in the striatum with time. Moreover, the signal was stable up to 60 days. This study demonstrates indisputable conceptual advantages of reporter genes over SPIO for cell tracking and long-term biodistribution. Reporter genes allow to precisely track stem cells, with high image resolution in any period. Reporter genes are also used in nuclear medicine imaging. PET/SPECT reporter genes' function is similar to MRI reporter genes, with the difference that PET/SPECT also requires administration of reporter probe containing radioisotope. When reporter gene is introduced into cells intended for transplantation, its product accumulates within the cell. Next, the reporter gene's product interacts selectively with radioactive molecules delivered in the reporter probe. Wu et al. (2013) used a reporter gene-probe system HSV1-tk-131I-2′-fluoro-2′-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil (^{131}I-FIAU) to analyze the biodistribution of bone marrow stem cells in rats with middle cerebral artery occlusion. Cells were transplanted via several methods, local injection, ventricular injection, carotid artery injection, and tail vein injection, and monitored by SPECT at 2-, 8-, and 24-h time points after probe injection. Direct, local injections to the brain led to more significant radioactivity accumulation than other delivery routes. In the beginning, whole-body SPECT imaging showed a low-level signal accumulation in the brain, which increased gradually over time. An unquestionable advantage of PET/SPECT reporter gene imaging is the possibility of efficient cell tracking and biodistribution assessing, as long as cells are viable and express reporter gene product. It allows for short- and long-term imaging, whenever a probe with the radioactive molecule is

delivered. Reporter genes MRI and radionuclide imaging are a grand promise for long-term cell tracking and viability; however, they have not been used in humans so far because of ethical considerations.

6.3.4 Multimodal Imaging

There is no single ideal tool for imaging transplanted stem cells. Each modality has its strengths and drawbacks. Therefore, currently, two or even more imaging techniques are often combined to complement one another. For example, imaging of living cells with BLI may be joined with MRI SPIO imaging. When BLI provides the perfect imaging cell viability and global biodistribution tool, MRI SPIO or reporter genes complement imaging with high-resolution anatomical structures for long-term tracking. Daadi et al. (2009) used a double fusion (DF) reporter gene that stably expressed enhanced green fluorescent protein (eGFP), firefly luciferase for BLI, and SPIO for MRI imaging of transplanted (hNSCs) in ischemic rat brain. BLI enables to noninvasively follow and quantify the survival and potential excessive growth of grafted hNSCs. In all animals, BLI signals were most robust at the first time point after transplantation (day 2) and remained relatively stable for 8 weeks. MRI allowed graft localization as hypointense areas in the striatum and the stroke zone's visualization as a hyperintense region in the striatum and cortex on T2-weighted images. MRI signal showed stable signal throughout the 4 weeks. The combination of these two techniques allows for assessing cell viability and long-term high-resolution tracking. Also, a variety of MRIs with radionuclide imaging shows great potential. Tang et al. (2015) synthesized MRI/SPECT/fluorescent trimodal probe (^{125}I-fSiO4@SPIO) to track IC and IV transplanted mesenchymal stem cells (MSC) into rat stroke model. IC injections were administered to the contralateral hemisphere. Three days after the procedure, SPIO-labeled MSC was seen on MRI as a hypointense signal, migrating from the injection site to the lesion area along the corpus callosum. SPECT imaging showed that 35% of IC injected cells migrated to the lesion site, while 90% of IV injected cells remain trapped in the lungs 14 days after transplantation. The combination of MRI with PET/SPECT can be used for stem cell tracking in humans, and we assume that it will become more and more popular in future clinical trials. Also, reporter genes are used in multimodal imaging. Daadi et al. (2013) tracked engineered embryonic stem cell-derived NSCs with a triple fusion reporter gene in a rat middle cerebral artery occlusion model. These cells expressed monomeric red fluorescence protein, herpes simplex virus-truncated thymidine kinase for multimodal imaging, and SPIO for MRI. SPIO MRI allowed for 12 weeks of tracking and revealed a reduction of infarct size in transplanted animals compared to control. Moreover, fluorodeoxyglucose containing the radioactive isotope ^{18}F was administered to the animals. They used PET imaging to show that lesioned hemisphere's metabolic activity has increased by 40% from week 1 to week 5. After infusion of reporter probe [^{18}F] fluorohydroxymethylbutylguanine ([^{18}F]FHBG), the NSC-expressing HSV-ttk reporter gene enables to directly and specifically image the grafted cells in the brain based on detecting

phosphorylation and entrapment of [^{18}F]FHBG in the cells. Imaging signal was stable from week 4–8 after transplantation and correlated with the size of graft measured by MRI. Multimodal imaging will be an important aspect of future human preclinical and clinical research. While BLI allows for high-sensitivity long-term cell viability monitoring in laboratory animals, complementary imaging with MRI allows for better spatial resolution. In contrast, use of MRI/PET/SPECT reporter genes provides for greater sensitivity and sustained signal even after cell division.

6.4 Imaging of Cell Function

Imaging is a unique modality capable of visualizing transplanted cells' function and in this way to correlate it with other outcome measures such as behavior. Basic optical imaging of the brain poses significant difficulties due to its structural complexity, restricted access, limited light penetration, light absorption, and scattering (Aswendt et al. 2014). Recent developments in imaging of therapeutic cells' functions are usually based on a combination of genetic engineering and large-scale, real-time imaging techniques (Fig. 6.2).

Intravital microscopy (IVM) is a method for imaging cell functions within living animals (Pittet and Weissleder 2011; Masedunskas et al. 2012). It allows for dynamic, real-time monitoring of cells and biological processes within their in vivo microenvironments. IVM provides cellular and subcellular-level resolution at sub-second imaging time (Choo et al. 2020). A common approach to IVM is introducing specific reporter genes to the cells of interest by genetic engineering and performing cellular transplantation. Such reporter genes can remain continuously active when controlled by a constitutive promoter, like β-actin (Damdindorj et al. 2014). Its activity can also be regulated by environmental stimuli such as biochemical or transcriptional changes. For example, the reporter gene expression can be driven by activation of hypoxia-inducible factor-1α gene promoter, which occurs during stroke (Zhang et al. 2009). For brain studies, IVM requires an imaging window within the animals' skull combined with an appropriate readout system, like a multiphoton or confocal microscope. The cells' fate can then be microscopically traced by identifying reporter molecules' presence through the cranial window (Kim et al. 2012). A wide variety of molecules can be used as reporters, including fluorescent proteins, which allow to trace migration and observe cellular functions in real time (Radbruch et al. 2015). Expression of fluorophores by modified cells observed with IVM could prove that transplanted stem cell is performing its function, as it was shown in case of proliferation of single transplanted hematopoietic stem cell (Turcotte et al. 2017), modulation of the inflammatory response by transplanted NSCs (Lee et al. 2008), or delivery of antitumor agents by transplanted MSCs with their subsequent self-elimination (Shi et al. 2019). IVM could also be used to verify systemically transplanted cells' ability to cross the BBB, determine their tropism and homing ability within the brain, and trace their cellular interactions with the host. Jablonska et al. combined two-photon microscopy with intravital

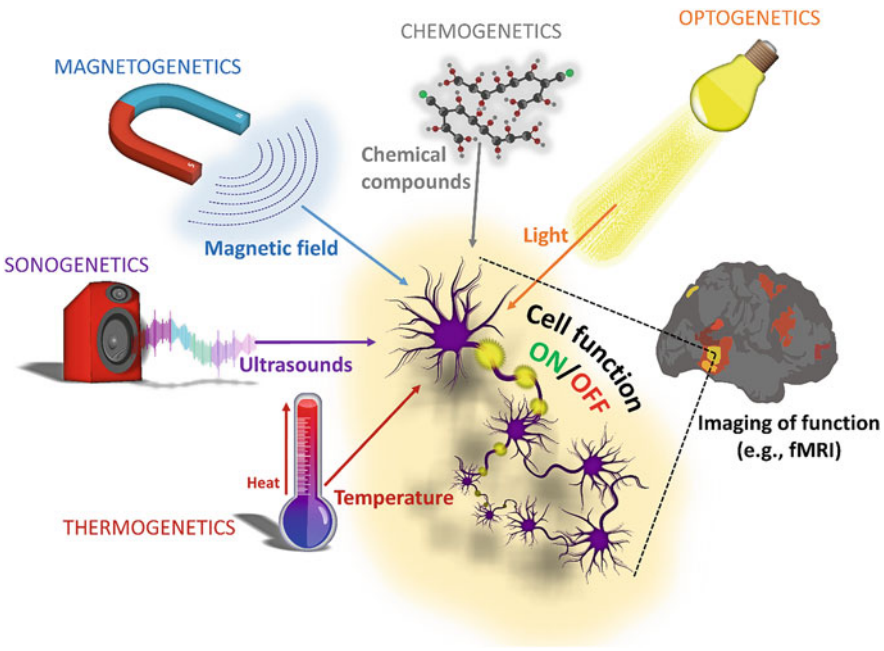

Fig. 6.2 Methods for modifying and imaging of cell function. Cell functions (e.g., neuronal firing in the brain) can be activated and deactivated by external stimuli. Such activation/deactivation can be monitored in real time with large-scale imaging techniques, such as fMRI (functional magnetic resonance imaging)

imaging demonstrating enhanced migration and adhesion abilities of IA transplanted, VLA-4 (very late antigen-4)-overexpressing glial-restricted progenitors in a mouse stroke model (Jablonska et al. 2018). Despite its enormous potential, intravital microscopy is limited by the restricted size of the cranial window. This limits the field of view, which could hinder the observation of migrating cells. Genetic engineering also has disadvantages, such as difficult translation from experimental to clinical scenarios and considerable safety concerns related to cell modifications.

Modern approaches allow not only to visualize but even control the functions of transplanted cells. Optogenetics is a powerful tool based on using light to control biological processes (Deisseroth 2011). The use of optogenetics requires the cell of interest to express light-sensitive optogenetic actuators, which are proteins that upon light stimulation modify the activity of this cell. Among the most popular actuators are opsins, such as ion-transferring channels like channelrhodopsins (ChRs), ion-transferring pumps like halorhodopsin (NpHR), or archaerhodopsin (Guru et al. 2015). Cells used in optogenetic applications can be genetically engineered ex vivo and then transplanted to the brain. Light activation delivered by optical fibers or micro-laser emitting diodes attached directly to the animals' skull triggers an instant response specifically in the optogenetically modified cells, allowing to study

cellular communication within neural circuits (Deisseroth 2015; Habibey et al. 2020). In turn, the activation of neural circuits by optogenetically activated cells confirms them being functional components thereof. Weick et al. demonstrated that upon optogenetic stimulation, embryonic stem cell (ESC)-derived neurons expressing ChR2 fire action potentials in vivo after transplantation to the CA3 region of the mouse hippocampus (Weick et al. 2011). Although initially used to control neuronal firing (Boyden et al. 2005; Deisseroth 2015), current optogenetic applications go well beyond just brain electrophysiology (Deisseroth 2011; Habibey et al. 2020; Entcheva and Kay 2020). Studies demonstrated effective optogenetic control over many cellular functions, such as Bax-induced apoptosis (Hughes et al. 2015), oligodendrocyte differentiation from glial progenitor cells (Ono et al. 2017), and Ras/Erk signaling pathway regulation (Toettcher et al. 2013). Optogenetics was also used to directly affect the properties of cells used in experimental therapy for neurodegenerative disease. Steinbeck et al. transplanted ESC-derived, NpHR-expressing mesencephalic dopaminergic neurons in a mouse model of Parkinson's disease. Engrafted cells fired action potentials and released dopamine, thus alleviating motor deficits. Interestingly, motor deficits were reintroduced upon optogenetically stimulated silencing of transplanted neurons (Steinbeck et al. 2015). Unfortunately, optogenetics suffers from some inherent limitations. Neuronal firing patterns induced by optogenetics may not necessarily reflect those occurring naturally (Rossi et al. 2015). Also, negative feedback from the optogenetic system may be difficult to interpret, as it may stem from insufficient actuator expression or inability to target the modified cell with the optic fiber.

Since upon optogenetic activation, grafted stem cells can affect regions distal to the transplantation site, additional techniques are required for large-scale assessments of cell functions in such therapeutic approaches. Functional magnetic resonance imaging (fMRI) measures the blood-oxygen-level-dependent (BOLD) signal to indicate oxyhemoglobin to deoxyhemoglobin ratio. Since neurons demonstrate significant metabolic activity requiring substantial oxygen supply, this ratio is correlated with neuronal activity (Ogawa et al. 1990). Recently, fMRI was coupled with BLI to monitor NSCs transplantation's effects in a mice cortical stroke model. While BLI confirmed cellular graft survival, fMRI demonstrated that transplanted cells stabilized functional connectivity within sensorimotor networks eliminating hypersynchronicity, which is a hallmark of mild cortical strokes (Minassian et al. 2020). Still, it is worth noting that since the BOLD signal is dependent upon blood oxygen and not the activity of any particular cell, fMRI alone cannot be used to study the functions of single transplanted cells.

The optimal system for studying functions of transplanted cells would enable external control over cellular graft with large-area imaging of its impact on the host. Optogenetic functional MRI (ofMRI) is a novel approach that combines simultaneous optogenetic activation of neurons with global fMRI brain readouts (Lee et al. 2010). The main advantage of ofMRI over traditional electrophysiology is that it allows studying in living animals, in real-time communication between distal brain regions, such as the vestibular nuclei and the sensorimotor cortex (Leong et al. 2019), the cerebellar cortex and the forebrain (Choe et al. 2018), or the thalamus and

the orbitofrontal cortex (Weitz et al. 2019). Therefore, ofMRI can be an indirect marker of transplanted cells' functionality, as visible by changes even in remote regions in the hosts' brain (Weitz and Lee 2016). It was shown that induced pluripotent stem cell-derived, optogenetically activated ChR2-expressing neurons transplanted into the rats' striatum generated both local and remote fMRI signals. Optogenetic stimulation of cells in the striatum affected BOLD signal not just within the striatum, but also in the hippocampus and other downstream brain areas (Byers et al. 2015).

In addition to widely used optogenetics, recent years brought progress in chemogenetics – techniques of precise control over cellular activity by chemically engineered molecules. Its combination with large-scale imaging techniques like fMRI could be an attractive approach in future stem cell-based therapies for stroke. One of the most widely used chemogenetic setups relies on designer receptors exclusively activated by designer drugs (DREADDs) in which designer G protein-coupled receptors are presented on cells and are then selectively activated by externally delivered molecules (such as clozapine-N-oxide) (Dobrzanski and Kossut 2017). Numerous studies showed that DREADDs enable spatiotemporally precise control over various cellular functions such as neuronal firing (Armbruster et al. 2007), microglia activation and inhibition (Grace et al. 2018), or excitotoxicity (Blázquez et al. 2015). Interestingly, in the context of stem cell-based therapies, hM3Dq DREADD increased proliferation and differentiation of oligodendrocyte progenitor cells responsible for post-lesional remyelination of axons (Mitew et al. 2018). DREADDs were already successfully combined with fMRI to study the functioning of complex brain networks in vivo. Peeters et al. have shown that kappa-opioid receptor DREADD-induced inhibition of the right anterior cingulate area affects BOLD signal in connected brain regions of both hemispheres, such as the sensory cortex and thalamus (Peeters et al. 2020). Unfortunately, chemogenetics is based on exogenously administered pharmacological agents such as clozapine, which can have numerous endogenous targets. Therefore, its administration could trigger brain responses that are detectable by fMRI (Garcia et al. 2015), but not necessarily related to the activation of DREADD.

Finally, three more modern techniques for imaging and modifying transplanted cells' functions are worth mentioning: firstly, sonogenetics, which uses ultrasound (US)-sensitive proteins to control cell properties. In a recent study by Wu et al., mice were transfected in vivo with plasmid encoding modified protein prestin, an electromechanical transducer present in the mammalian auditory system. The expressed protein was activated by transcranial, 0.5-MHz US stimulation, as indicated by triggering in transfected brain cells the expression of c-Fos, a marker of neuronal activity (Wu et al. 2020). The second technique is magnetogenetics, which is based on magnetoreceptors – proteins responsive to magnetic stimulation. Dufor and colleagues investigated cryptochrome magnetoreceptors, which activate upon repetitive transcranial magnetic stimulation at low intensity. Magnetic stimulation of cryptochrome-expressing brain cells led to upregulation of genes involved in neuronal repair and modulation of neurotrophin signaling, thus contributing to axonal growth and regeneration in a post-lesional mice brain (Dufor et al. 2019). The final

method is thermogenetics, which uses thermosensitive transient receptor potential (TRP) cation channels to control cells' properties. As shown by Munshi et al., heat-sensitized neurons expressing TRPV1 responded to heat generated by alternating magnetic fields in living mice. Stimulated neurons fired, activating complex neuronal circuits, thus triggering corresponding behavioral responses, such as ambulation, rotation around the body axis, and freezing of gait (Munshi et al. 2017).

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Nanomedicine-Mediated Stem Cell Therapeutics in Stroke

7

Namrata Sangwan and Pramod K. Avti

Abstract

Stroke is one of the key factors of mortality and morbidity across the globe. According to the World Health Organization (WHO), 15 million individuals suffer from stroke every year, of which 5 million deaths and the remaining 5 million remain permanently disabled. Due to its poor prognosis and even after knowing the seriousness of this neurological disease pathologically, tissue plasminogen activator (tPA) is the only approved FDA agent for ischemic stroke. There is no alternative effective therapy for stroke and only a few preventive measures are often considered to enhance the patient's quality of life. In view of this, regenerative medicine with stem cell therapy is one of the emerging areas to promote neuroregeneration. The use of nanotechnology mediates the regulation of stem cells, an emerging combinatorial approach, for stroke care in recent times. This combinatorial approach provides a versatile platform for stroke therapy by enhancing the ability of stem cell proliferation, migration, and differentiation leading to neuroregeneration. When provided at an ideal dose while taking into account the patient's age, sex, and other comorbid diseases, the combinatorial theranostics of nanoparticles influencing stem cell response might help reduce infarct volume. This chapter focuses on the present status of nanomedicine-mediated stem cell regulation for stroke therapeutics.

Keywords

Clinical studies · Ischemic stroke · Nanoparticles (NPs) · Preclinical studies · Stem cell-based therapies

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Abbreviations

ASA	American Stroke Association
AVM	Arteriovenous malformations
BBB	Blood-Brain Barrier
BDNF	Brain-derived Neurotrophic factor
CCI	Charlson Comorbidity Index
CT	Computed Tomography
CTA	Computed Tomography Angiography
CTP	Computed Tomography Perfusion
CVA	Cerebro-Vascular Accident
ESC	Embryonic Stem Cells
FDA	Food and Drug Administration
GDNF	Glial cell-derived neurotrophic factor
IA	Intra-arterial
IC	Intracerebral
IGF	Insulin-like growth factor
IL	Interleukin-6
IPSC	Induced Pluripotent Stem Cells
IT	Intrathecal
IV	Intravenous
LACI	Lacunar Infarction
LIF	Leukemia Inhibiting Factor
MCAO	Middle Cerebral Artery Occlusion
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cells
MWCNT	Multi-Walled Carbon Nanotubes
NINDS	National Institute of Neurology Disease and Stroke
NPSC	Neural stem cells/progenitor stem cells
NSC	Neural Stem Cells
OCSP	Oxford Community Stroke Project Classification
PACI	Partial Anterior Circulation Infarct
PAI-1	Plasminogen Activator Inhibitor
PEDF	Pigment Epithelial Derived Factor
PEG	Polyethylene Glycol
PLGA	Poly D, L-lactic acid-co-glycolic acid
ROS	Reactive Oxygen Species
rTPA	Recombinant tissue plasminogen activator
SVZ	Subventricular Zone
SWCNT	Single-Walled Carbon Nanotubes
TACI	Total Anterior Circulation Infarct
TGF	Transforming growth factor
TIA	Transient Ischemic Attack
tMCAO	Transient Middle Cerebral Artery Occlusion

TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

7.1 Introduction

According to WHO, the incoming epidemic of the twenty-first century is referred to as "stroke" which is the interruption of blood flow in the blood vessels of brain. In the 1970s, WHO described stroke as a neurological condition that sustains or can be disrupted within 24 h of causing death. Stroke is the most prevalent chronic acquired disability, with worrisome signs that occur every 5 seconds globally, according to the WHO. After cancer and myocardial infractions, it is the second biggest cause of death globally (Singh et al. 2020).

In brain stroke victims, different events, such as neural cell apoptosis at the molecular level and, physical disability (limb dysfunction), paralysis or slurred speech, etc., as the symptoms are observed (Chugh 2019). Usually, one-fourth to half of the stroke survivors are affected with some form of impairment. Ischemic stroke, hemorrhagic stroke, and transient ischemic attack (TIA)/mini-stroke are other types of strokes (Chugh 2019; https://www.neurogenbsi.com/stem-cell-treatment-for-stroke-patients#:~:text=Stem%20Cell%20Treatment%20for%20stroke%20has%20increased%20the%20hope%20of,mesenchymal%20stem%20cells%20promotE%20repair; Martínez-Garza et al. 2016; https://www.medicinenet.com/stroke_symptoms_and_treatment/article.htm; Benjamin et al. 2017) (Fig. 7.1). Every year, 15 million people worldwide suffer from stroke, with 5 million deaths, and another percent of whom remain permanently disabled. The most frequently observed stroke is ischemic (87%), followed by a hemorrhagic stroke (20%) (Virani et al. 2021; Centers for Disease Control and Prevention 2018).

The etiological components of stroke are associated with hypertension, smoking, excess use of alcohol, obesity, migraine, carotid stenosis, atrial fibrillation, diabetes mellitus, brain aneurysm, and atherosclerosis (Reis et al. 2017). Along with the

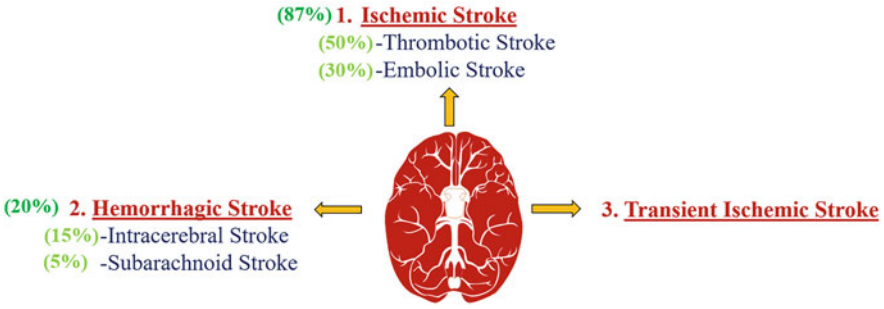

Fig. 7.1 Type of stroke

above-mentioned factors, age and sex of a person also imply a critical role in stroke etiology. The chances of stroke increases with the advancing age (age > 55 years), and men are more likely to suffer at younger ages than women. However, the fatality of stroke is higher in women, especially who are on birth control pills (Bang et al. 2016).

It is also observed that cardiovascular diseases such as coronary heart disease, cardiomyopathy, heart failure, atrial fibrillation, and arteriovenous malformations (AVM) can cause blood clotting in the brain and later develop into stroke (Boese et al. 2018; Kawabori et al. 2020). Hypercholesterolemia causes the deposition of fatty acids in the arteries of the brain causing the formation of brain plaques that can also result in stroke (Kawabori et al. 2020). Excess smoking causes damage to the blood vessels and increases the blood pressure along with reducing the oxygen to reach the body tissues which further damages the nerve cells leading to stroke (Savvina and Petrova 2020).

Much effort is made over the recent decade to investigate the possible use of stem cell treatment as a stroke therapy strategy. Based on their sources, stem cells are of various types, i.e., embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), hematopoietic stem cells (HPSCs), adipose-derived stem cells (ADSC), etc., which are transplanted as either an autograft, allograft, or xenograft (https://www.neurogenbsi.com/stem-cell-treatment-for-stroke-patients#:~:text=Stem%20Cell%20Treatment%20for%20stroke%20has%20increased%20the%20hope%20of,mesenchymal%20stem%20cells%20promotE%20repair; Anderson et al. 2016; Kurozumi et al. 2005; Macrae and Allan 2018; Sinden et al. 2012). Stem cells due to their remarkable ability of differentiation and release of various trophic factors (such as vascular endothelial growth factor (VEGF), brain-derived growth factor (BDNF), or transforming growth factor (TGF)) play an essential role in treating stroke by promoting angiogenesis, neurogenesis, and synaptogenesis (Sinden et al. 2012).

These stem cells not only have increased the hope of curing the disease, but also ideal for stroke treatment as they can be produced from one's bone marrow, having lesser rejection and ethical issues. Because of their safe and effective treatment methods, they are widely recognized and accepted as an innovative agent for stroke treatment. To overcome the challenges in stroke theranostics, a combinatorial approach via the use of nanoparticles (NPs) like metallic NPs, polymeric NPs, carbon nanotubes (CNTs), etc., with stem cell transplantation is an emerging strategy for therapy. The regulation of stem cell therapy through nanoparticles provides appropriate stimulus conditions for the stem cells to proliferate and differentiate on a larger scale. Although the combinatorial approach of stem cell therapy and nanotechnology for this neurological disorder is still in nascent stage, it has great potential for regeneration after stroke.

In this chapter, we emphasize on some of the recent approaches regarding the use of nanoparticles-mediated stem cell regulation for stroke therapy. The major focus of the chapter is on how different types of nanoparticles, depending on their physico-chemical qualities and nature, regulate the stem cells in terms of effective dose, mode

of administration, and processes of differentiation and regeneration in reducing the infarct volume and improving neurological outcomes.

7.2 Current Status of Stroke

Often known as "stroke" is cerebro-vascular accident (CVA)/brain attack. Stroke is considered as an impediment in the functioning of the brain caused by the obstruction of cerebral blood flow leading to an irreplaceable neurological deficit in the patient's body (https://www.medicinenet.com/stroke_symptoms_and_treatment/article.htm). The blood vessels and tissues supplementing the necessary nutrients and oxygen to the brain and other parts halts because of the interruption of blood supply in the brain, resulting in neurological impairments that leads to serious effects such as loss of control, numbness, speech difficulty, etc. (Hankey 1999). A stroke can last up to several hours for a long time, but a smaller stroke is typically referred to as a ministerial stroke or transient ischemic attack (TIA), due to the momentary occlusion of blood flow (Martínez-Garza et al. 2016).

Stroke is one of the primary causes of chronic disability and mortality, with 102 million disability-adjusted life years lost each year. The second major cause of mortality and the third major cause of injury worldwide due to stroke (Gryskiewicz et al. 2008). The USA stroke data shows 87% ischemic and 10% hemorrhagic stroke (Chugh 2019). Banerjee et al. in 2001 estimated the crude prevalence rate of stroke in India to be 147/100000 and the annual incidence rate as 36/100000. The WHO has calculated that a stroke occurs every 5 seconds (Laso-García et al. 2019). The accounts for 2005 are approx. 10% of all deaths worldwide, making it the second most common cause of death (Moskowitz et al. 2010). A stroke occurs every 40 seconds in the United States, which means 2160 strokes per day. Nearly 1 out of every 16 Americans die of stroke (Sarikaya et al. 2015).

The American Stroke Association (ASA) has postulated an abbreviated form of manifestation to be observed in a person which is termed as **'**FAST'; stands for Facial drop**;** Arm weakness**;** Slurred speech, and Time of onset. One more way to remember the sign and symptom of a stroke is the '6S' method, i.e., Sudden (Sudden start of symptoms); Slurred speech (not clear speech); Side weakness (weakness in the face, arm, or leg); Spinning (vertigo); Severe headache (rush to the hospital in seconds) or 'BEFAST' method, Balance loss; Eye visiondim/loss; Face drop; Arm weakness; Speech slurdness (Fig. 7.2) (Chrostek et al. 2019). The involvement of neurological entities may range from mild motor deficits to the involvement of many other functions such as emotional, speech, and language dysfunction, memory or intelligence, sensorimotor perceptional, lack of coordination (ataxia), and other symptoms associated with stroke (Gao et al. 2018).

The pathophysiology of stroke involves various processes that work all together in a coordinated manner to exert psycho-physiological impacts. This process involves energy failure, loss of cellular ion homeostasis, the formation of arachidonic products, cytokine-mediated cytotoxicity, complement activation, and disruption of blood-brain barrier (BBB) activates the glial cells, and infiltration of

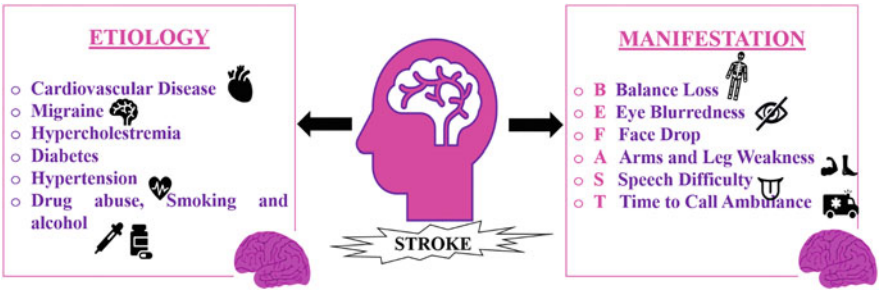

Fig. 7.2 Etiology and manifestation of stroke

leukocytes (https://www.radiologyinfo.org/en/info.cfm?pg=stroke#:~:text=Because%20treatment%20depends%20on%20the,ultrasound%2C%20echocardiography%20or%20cerebral%20angiography).

The morphological characteristics include initial cellular swelling, distortion of a nuclear and cytoplasmic organelle, and evading out of cell content of extracellular space a process termed necrosis. Along with necrosis apoptosis also plays an important role in stroke. Apoptosis only damages the particular target cells and not the neighboring cells, hence minimizing the infection/inflammation caused by necrosis. Apoptosis occurs in two pathways either intrinsic or extrinsic (Rowley and Vagal 2020). Energy failure is induced by the cellular pathophysiological process, which depolarizes the neurons. By the activation of the glutamate receptors, there is an increase in the intracellular Ca^{2+}, Na^{2+}, and K^{+} ions which are released extracellularly. Thereafter, the water shifts intracellularly causing edema, generating free radicals leading to the cell membrane, DNA, and mitochondria disruption. Thus, results in the activation of apoptotic pathways through caspase-9 and 3 activity (Young and MacDougall 2019). Activation of transcription factors such as JNK, P-38, NFκB, and AP-1 also causes the glial cells, endothelial cells, and leukocytes to regulate inflammatory mediators leading to cell death/apoptosis (Zakrzewski et al. 2019). The whole coordinated and integrated pathophysiology will lead to invasion of leukocytes by upregulating the endothelial adhesion molecules and escalating the glutamate excitotoxicity, oxidative stress, lipid peroxidation, inflammation, BBB dysfunction, and leukocyte infiltration (Misra et al. 2012; Chrostek et al. 2019) (Fig. 7.3).

Nowadays various advancements have been made in brain imaging of stroke used in diagnostic scans to easily demonstrate the type of stroke (hemorrhagic or ischemic stroke) associated with patients depicting the different stages i.e., acute, sub-acute, and severe. Usually, patients of ischemic stroke in clinical trials have to go through a computed topography (CT) scans or magnetic resonance imaging (MRI) scans and identify the penumbral and core (umbra) region; also, to see how much tissue has been damaged and can be recoverable (Barzegar et al. 2019).

Imaging techniques, for instance, CT scan and MRI reveal anatomical, physiological, and molecular images; also, single-photon emission computed tomography (SPECT) and positron emission tomography (PET) show distinct images to do

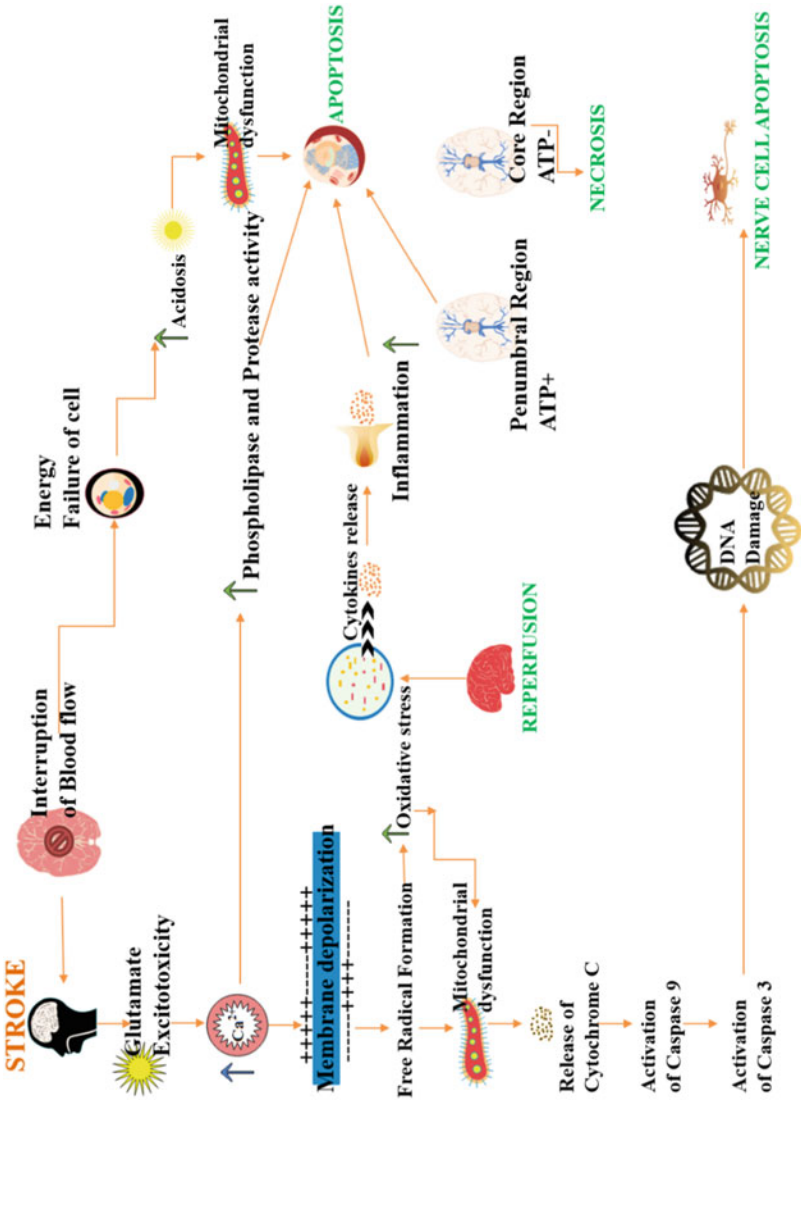

Fig. 7.3 Pathophysiology of stroke

diagnosis in biomedical research. Using these imaging techniques, one can readily figure out the brain networks involved in cognitive activities, identify molecular and signaling pathways (Medhi et al. 2014).

MRI imaging for the penumbral region is also available for the study of rodent stroke where they tracked the loss of penumbra in the brain region; this resulted in obtaining additional informative data to successfully analyze the infarct sites (Kim 2019). Typically it is considered that the very first step in assessing the stroke is either by a CT scan or MRI. CT scan combines X-ray equipment with a computer to produce various images of the head (inner view). Generally, physicians use a CT scan of the head to determine which type of stroke is diagnosed. Further, in a multi-modal approach, CTA is also performed alongside CT, wherein the contrast agent perfused through IV enhances the characterization and detection of infarct region in the brain. In another approach, computed tomography perfusion (CTP) produces images that detect the blood flow at the same time (Kim 2019). Hence, physicians usually recommend CT, CTA, and CTP to experience the best diagnosis. MRI is a powerful technique of magnetic field, pulses of radio frequency, and computer to produce a detailed image of the internal anatomical structures; and the MR image is used to analyze the cerebral vessels via MR angiography (Medhi et al. 2014; Bansal et al. 2013). On the dependence of the time of manifestation onset, the aid in management is carried out either when the patient is being analyzed within the first 6 h of manifestation or not. Whereas, two management approaches were made either if the patient presents within the first 6 h or between 6 and 24 h of stroke manifestation. For manifestation within 6 h, CT scan is combined with CTA of the brain and neck to analyze the occlusion in large vessels; and for the manifestation between 6 and 24 h, CTP, diffusion-weighted MRI or MRI perfusion are used to figure out the occlusion in the anterior circulation of blood vessels as per the mechanical thrombectomy (Bansal et al. 2013). MRF shows similarity with quantitative MRI (qMRI) and helps in analyzing the realistic vasculature in mice. Also, a study has been conducted which developed a system to image the entire brain of mice at microscopic resolution and perfusion procedure by using the dual-modality technique (OCT - optical coherence tomography and CF - confocal microscope) combined with a vibrating blade sectioning for 3D reconstruction of the brain; diagnosing changes in microvasculature and white matter in the brain (Pouliot et al. 2017; Castonguay et al. 2015). Recently, a novel approach for the acquisition and analysis of magnetic resonance data, i.e., magnetic resonance fingerprinting (MRF) is studied in the realistic vasculature in mice. vMRF (a variant of vascular magnetic resonance fingerprinting) generally extracts the map considering three parameters, i.e., cerebral oxygen saturation, mean vessel radius, and cerebral blood volume (CBV) (https://www.healthline.com/health/stroke/drugs).

As a result, we may overcome the limiting therapy options in stroke patients by using multi-modal and multi-functional diagnostic detection techniques and contrast agents to follow the homing of stem cells at the specific affected region. Such emerging techniques might also solve multiple technical obstacles faced in stroke treatment by further the using nanoparticle regulated stem cell therapy.

7.3 Therapeutic Strategies

7.3.1 Drug Therapeutics

The role of drugs/medication in treating stroke typically works in different ways; few of them break up existing blood cells including platelets by acting on them or they prevent blood clotting in the blood vessels. Various anticoagulant and antiplatelet drugs are used in preventing stroke. Antiplatelet drugs, for instance, Plavix (clopidogrel) are used to prevent clotting of blood; aspirin is also given as an antiplatelet drug but the only limitation is the high risk of bleeding (Bliss et al. 2010). Anticoagulant (e.g., warfarin) is used to prevent the existing clots from getting bigger. Heparin is a fast-acting anticoagulant in comparison with warfarin which is slow acting. Dabigatran is also an indirect thrombin inhibitor that acts as an anticoagulant and is approved by the Food and Drug Administration (FDA). Other statin drugs, such as Lipitor and Crestor, are used to reduce the high cholesterol level in adults by reducing the risk of hypercholesteremia (O'Collins et al. 2017). Till now, the mainstay medical treatment for ischemic stroke is the IV thrombolysis of alteplase (rTPA) along with plasminogen activator (like streptokinase and urokinase), which leads to the promotion of thrombolysis. Therefore, FDA in 1996 approved the use of IV alteplase as the result of the National Institute of Neurology Disease and Stroke (NINDS) study. The study conducted by NINDS 2 trials was performed having 624 patients which are treated with alteplase (0.9 mg/kg) within 3 h of acute ischemic stroke (Nagai and Granger 2011). However, till date the most effective and suitable drug approved by FDA is rTPA, though different antihypersensitivities, antipyretic, and anticoagulant medicines are utilized in unison as possible stroke therapies (Rosso et al. 2019).

7.3.2 Stem Cell Therapeutics

Usually, stroke care turns out to be a bit cumbersome because of the catastrophic pathophysiology triggerings and delaying the recovery of BBB permeability. The central nervous system's (CNS) nerve cells have the poorest conceivable regeneration capability, which results in a substantial amount of degeneration. To overcome all these challenges in stroke theranostics and combinational cure, the use of nanoparticles mediated stem cell transplantation acts as an emerging curative possibility in the stroke treatment. Refer Tables 7.1 and 7.2 for various preclinical and clinical studies mentioned below in detail.

7.3.2.1 Treatment Conditions

Location of Infarct

Necrosis occurs due to an inadequate blood and oxygen supply to the affected area of the brain. It is usually termed as infraction and the resulting lesion is called as infarct (Seyman et al. 2016). Usually, an ischemic or cerebral stroke occurs due to the

Table 7.1 *In vivo* study

Species	Animal model	Stem cell	Dosage (No. of cells)	Route of Administration	Time window	Comorbid conditions	Outcome	References
Wistar rat	MCAO (focal cortical ischemia)	BMMNC	3×10^7	IV	90 min	Aging	Condition improved	Coelho and Giraldi-Guimarães (2014)
Wistar rat	MCAO	HUSC	1×10^7/kg	IV	120 min	Aging	Condition improved	Zhang et al. (2013)
Wistar rat	MCAO	BMSC	2×10^6	IA	24 h	Aging	Condition improved	Shen et al. (2007)
Wistar rat	MCAO	BMSC	3×10^6	IV	24 h	Diabetes type 1	No improvement	Chen et al. (2011)
SHR	MCAO (intracerebral hemorrhage)	BMSC	1×10^6	IC	24 h	Hypertension	Condition improved	Chen et al.
Long Evans	MCAO (neocortical)	BMMNC	4×10^6	IA	24 h	Aging	Condition improved	Breneman et al.
Rat	MCAO	Pig AD-MSC	2×10^6	IV	48 h		Sensorimotor improvement	Ding et al. (2017)
Rat	Endothelin	Human MSC	1×10^6	IC	Within 24 h		Improved motor function	Chen et al. (2014)
Rat	MCAO	hUCMSC	2×10^6	IC	1 week		Improved functional recovery, increased neurogenesis and angiogenesis	Kurozumi et al. (2004b)
Sprague-Dawley rat (female)	Embolic model	Neurotrophic factors	1×10^6	IV	6 h	Aging	Improved functional recovery	Dinapoli et al.
Sprague-Dawley rat (male)	MCAO (intraluminal suture)	Human iPSC	0.3×10^6	Intracortical	2 days	Aging	Reduction in infarct volume	Prasad et al. (2012)
Mice	MCAO	BMMNC	3×10^6	IA	24 h		Improved sensorimotor function, enhanced angiogenesis	Kalladka et al. (2016)
Mice	Photothromo-embiotic	Human BMSC	2×10^6	IV	24 h		Improved functional recovery, decreased lesion volume	Moniche et al. (2012)

Table 7.2 Clinical trial study

Stroke	Stem cell	Source of stem cell	Dosage (No. of Cells)	Route of Administration	Transplantation time	Country/ no. of patients	Outcome	References
Acute ischemic stroke	Autologous	BMMNC	4.6×10^8	IV	1–3 days	USA/10	Improvement in neurogenesis	Savitz et al. (2011)
Acute ischemic stroke	Autologous	BMMNC	1.6×10^9	IA	5–9 days	Spain/10	No differentiation or neurological development	Magnusson et al. (2014)
Sub-acute ischemic stroke	Autologous	BMMNC	2.19×10^8	IV	2–4 weeks	India/11	Improved functional recovery found in early treatment	Balseanu et al. (2014)
Sub-acute ischemic stroke	Allogenic	Fetal neural cells	2×10^8	IT	4 months	Russia/1	Treatment showed 33% in Karnovskii score	Sharma et al. (2014)
Chronic ischemic stroke	Autologous	BMMNC	6×10^7	IT	4 months	India/14	Show recovery but later on effected by hemorrhage	Plane et al. (2008)
Chronic ischemic stroke	Allogenic	Fetal neural cells	2×10^7	IC	2 months-1 years	UK/23	Upper limb function recovered	Sims et al. (2009)
Chronic ischemic stroke	Allogenic	Fetal neural cells	$2,5,10,20 \times 10^6$	IC	1–4 years	UK/11	Increased neurogenesis	Chou et al. (2006)
Chronic ischemic stroke	Autologous	CD34+	$3–8 \times 10^6$	IC	6 months-5 years	Taiwan/ 15	Significant recovery, improved angiogenesis	Choi et al. (2016)

clotting of blood, plaque formation or atherosclerosis, etc. Sometimes the blockage maybe because of occlusion of blood vessels, thrombus, embolus, or narrowing of arteries which results in the infarct into the ipsilateral region of the brain (Roy-O'Reilly and McCullough 2018). These different types of infarcts lead to varied symptoms which is assessed by blocked arteries; revealing which part of the brain is affected by infarction.

The Oxford Community Stroke Project Classification (OCSP) classified infarcts on the extent of stroke, affected area, and causes into three types – total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), and lacunar infarction (LACI) (Popa-Wagner et al. 2014). In the case of TACI and PACI, the anterior side of the brain circulation gets affected whereas, in LACI the small arteries that provide blood to deep brain parts get occluded (Barthels and Das 2020). Hypertension, the period of occlusion into small arteries, and reperfusion will correspond with the development of the infarct, are multiple physiologic variables that impact the infarct size. Several other physiological factors also influence the size of the infarct such as temperature, acidification, or glucose content (Benjamin et al. 2018). In the *in vivo* animal models, MCAO is found to be the most promising method to analyze the size of the infarct, factors affecting infarct, and its associated treatment strategies (Lee et al. 2016). The infarct volume measure is the effectiveness of the evaluation of treatment for stroke in animal model studies. Infract size and locations are the correlating parameters that are majorly associated with risk factors and outcomes (Chen and Li 2020). The different shapes of the infarct results were analyzed in the clinical trials, for instance, 63% ovoid/spherical, 12% slab, 7% stick, 17% multicomponent in which the infarct size is correlated with the same vascular risk factors among all different infarct shapes and sizes (Benjamin et al. 2018). In an *in vivo* study, the location of infarct in Long Evans rats is found neocortically which improved upon transplantation with bone marrow-derived mononuclear cells (BMMNC) cells resulting in a reduction in infarct size (Lee et al. 2016). Whereas on Sprague-Dawley male rats, the location of the infarct is found in the subcortical and neocortical region which later on treating with human iPSCs reduced the infarct volume (Tatarishvili et al. 2014). The location of infarct volume in Sprague-Dawley female rats is present in the neocortical region which shows recovery after treating with MSCs (Chen and Li 2020). A common index for the assessment of ischemic stroke followed by focal cerebral stroke is infarct volume which is postulated by brain imaging, CT scan or MRI and helps in determining the extent of infarct and its associated area of the brain. In acute patients, the area is quietly diminished but in chronic patients, the infarct is large having a gliotic rim around the infarct volume (Sarmah et al. 2017).

A clinical trial study by Brott et al. (1989) and Caviness et al. (2002) that effectively measured the infarct volume after 1 week of ischemia using CT showed average infarct sizes of 55 cm^3 and 3.1–256 cm^3 (Naqvi et al. 2020). The analysis of infarct size in a controlled clinical trial studied that the mean infarct size (expressed in % of brain volume) was found to be 47 + 28.3 cm^3 which is equal to 8% (in one cerebral hemisphere) and the mean volume of infarct ranged from 5.3 cm^3 (0.9% of the hemisphere) to 124.8 cm^3 (in one hemisphere) (Alkaff et al. 2020). Meanwhile,

in the study of different strains of rats, an average value ranged across species of rats (Wistar, Sprague-Dawley) 53.6 mm^3 in mice to 47.4 cm^3 in human beings. The average infarct size is 112.5 mm^3 in long Evan rats to 197.6 mm^3 in hooded Wistar rats. Therefore, it is concluded that infarct volume in one cerebral hemisphere *in vivo* models is 30% (volume) and 29% (% of the hemisphere) whereas in clinical trials mean is 10 and the median is 7.

When treated in ischemic stroke brain, the MSC-derived extracellular vesicles showed repaired neurovascular unit and reduced the size of the infarct in the cerebral area with a marked increase in neurogenesis, microglial cells, and astrocytes in MCAO occluded rats (Davis et al. 2021). A sensitive agent polyoxometalates nanocluster in association with NSCs for reactive oxygen species was developed to reduce oxidative stress, apoptosis suppression, edema, and decreased volume of infarction (up to 40–50%) in the acute ischemic brain (Gao et al. 2018). One among the most popular successfully used biodegradable polymers is poly (lactic-co-glycolic acid) (PLGA) (Naqvi et al. 2020). Using plasma polymerized allylamine-treated poly (D, L-lactic acid-co-glycolic acid) scaffolds, neural stem cells were cultured, which later became neurons and astrocytes, improving angiogenesis and decreasing the amount of infarction (Alkaff et al. 2020). Curcumin-encapsulated PLGA nanoparticles display remarkable effects on neural stem cells in both *in vivo* and *in vitro* studies, resulting in reduced infarct size and enhanced neurogenesis in the brain hippocampal area. Thus, PLGA can be utilized as a carrier and for the encapsulation of stem cells and drugs because of its attractive properties. The therapeutic benefit of immobilizing substance P into self-assembled proteins with MSC through biological activities in an ischemia hind limb model indicated a reduction in the infarct zone, tissue regeneration, and enhanced angiogenesis (Rikhtegar et al. 2019). In stem cell therapy, the amine-modified single-walled carbon nanotubes (SWCNTs) also serve as a scaffold and it is also incredible to know that carbon nanotubes alone are capable of reducing the size of the infarct by improving the resistance of rat brain ischemic stroke (Moeini et al. 2018). Hwang et al. in 2012 in the study of carbon nanotubes with stem cells is prescribed as a promising path to neurodegenerative disease regeneration of nerve tissue by incorporating in NSCs. In terms of labeling neural stem cells, cationic polymeric micelles concluded that neural stem cell micelles displayed greater effectiveness, protection, and reliability in reducing the infarct size in the *in vivo* stroke model using MRI imaging technique for study (Zhao et al. 2016). Similarly, after cerebral acute ischemia infarction with polymeric nanoparticles, used supermagnetic iron oxide filled with cationic polymerosomes regulates neural stem cells to minimize the size of infarction in the cerebral cortex. It is also revealed that retinoic acid loaded with polymeric nanoparticles decreases the size of the infarct by increasing the vasculature regulation of differentiation and survival of neural stem cells when crossing the neuroprotective BBB (Ferreira et al. 2008).

Age and Sex Effects

Advancing age in males as compared to females is a recognised and non-modifiable risk factor which causes complex and additive effect that accounts to stroke. Aged

persons suffering from stroke have high mortality, fewer chances of recovery, and morbidity as compared to the younger ones. In the advancing age risk of stroke is higher in males instead of females (Naqvi et al. 2020). The disease of aging, i.e., stroke shows interactive results with the sex of the individual also as men show higher incidence whereas women show higher prevalence. In childhood/early adulthood men show higher incidence; in middle age rate of stroke increases in women and after that mortality risk increases in men at the age of 65 or more (Lee et al. 2016; Chen and Li 2020). Changes in brain microvasculature, such as capillary width, density, thickness, and changes in the composition of the vessel wall in arteries and capillaries are all associated with aging process. These modifications together with metabolic changes manifest reduction in cerebral blood flow and an insufficient blood supply to areas of the brain. Concessions in oxygen and glucose supply to the brain can harm neurons and have an impact on cognitive decline (Moeini et al. 2015). Also, the reduced cerebral bold flow due to aging in the vascular wall structure of the brain leads to loss of lesion formation in the white matter of the brain, and causes an alteration in neurovascular coupling in aged mice (Pouliot et al. 2017).

The effect of age on Wistar rats having focal cortical ischemia shows improved results in a reduction of infarct size (Hallbergson et al. 2003); similarly, the study of ischemic stroke model of MCAO in Sprague-Dawley rat male and female respectively showed induced functional recovery in the reduction of infarct size in neocortical and subcortical located infarct (Sarmah et al. 2017). In terms of shreds of evidence for sex and age effects in experimental models of stroke (specifically ischemic stroke), the *in vitro* stroke models cause major harm to the male neurons instead of females when they performed modeling using oxygen glucose deprivation (OGD) in which glial and neural cells were cultured without glucose and oxygen causing energy failure and loss of cerebral blood flow (Alkaff et al. 2020). In small *in vivo* rodent models results show that females are less affected as compared to males in cerebral ischemia, which indicates that estrogen is ischemic resistant in females (Chan et al. 2017). In clinical terms, the sexual dimorphism of stroke highly depends on age. In *in vitro* studies, middle-aged, larger infarcts, and poor outcomes occur in females in comparison with males (Popa-Wagner et al. 2014; Nucci et al. 2015). This is due to that the onset of reproduction senescence in mice increases the sensitivity of stroke in middle-aged female mice due to the loss of estrogen. But the elderly male and female mice have some same-sex hormonal level so the size of stroke equalizes. So, it clearly shows the interactive effect of stroke experimental pathophysiology, indicating their influence in clinical studies (Barthels and Das 2020; Nucci et al. 2015). The effective role of advanced aging in association with oxygen level causes a substantial increase in hypoxia in brain tissue, leads to neuronal death and impairment in cerebrovasculature because of the advent of reactive oxygen species (ROS) and calcium dyshomeostasis (Arora et al. 2012). *In vitro* (cellular therapies) show reduced ability in old age concerning safety when it comes to cell delivery because aged males have a cardiovascular system less tolerable for the large cell intravascular cell loading. Meanwhile, the allogenic cell implants restrict older patients concerning reduced recovery and getting the risk of

tumors (Zhang et al. 2020). The mediation of stem cells with nanoparticles also shows improved results in the male mice of 8–10 weeks old without loss of cell viability or proliferation, adipose-derived stem cells were effectively labeled without loss of cell viability or proliferation, adipose-derived stem cells were effectively labeled with SPIO NPs (Rice et al. 2007). The impregnation of CNTs-SVZ-NPCs (Carbon nanotubes-Subventricular Zone-Neural progenitor cells) in MCAO induced male Sprague-Dawley rats of 10 weeks weighing approx. 300–330 gm, enhanced rats neurological behavior and decreased infarct volume (Moon et al. 2012a). The MCAO-occluded male SWISS albino mice (∼28–32 g, 6 weeks old) were IV administered with squalenoyl adenosine nanoparticles (SQAd NPs) which showed improved neurological outcomes (Gaudin et al. 2014). Thus, the above studies suggest that the administration of nanoparticle-mediated regulation of stem cells improves the behavioral outcomes irrespective of age- and sex-biased effect of stroke.

Comorbidities

A number of multi-morbidities/comorbidities are associated with the onset of stroke, for instance, hypertension, diabetes, atrial fibrillation, atherosclerosis, smoking, drug abuse, obesity, migraine, hypercholestremia, brain aneurysms, carotid stenosis, etc. (Popa-Wagner et al. 2014). The comorbidities are analyzed and estimated via the Charlson Comorbidity Index (CCI) which act as a prognostic indicator of ischemic stroke. Multi-morbidities affect the survival of patients having stroke and shown the worst recovery after treatment (Nucci et al. 2015).

It is estimated that 6% of patients had no comorbidities, whereas 40% suffering from an ischemic stroke is associated with 2/3 of diseases. Approx. 75% who had a stroke are associated with cardiac comorbidities including atrial fibrillation, cardiomyopathy, endocarditis, etc. Ischemic stroke in 40% of patients shows diabetes mellitus which doubles the risk of stroke which causes an effect in the outcome of medical rehabilitation and recovery (Nucci et al. 2015).

In a study of a clinical trial, a total of 264 patients with acute stroke has analyzed that successful rehabilitation of 33.7% of patients has been undertaken who have comorbidities of atrial fibrillation, diabetes mellitus, and approx. 28.5% of diabetes mellitus patients also had successful results concluding that patients with no comorbidities are achieved better recovery than those of associated comorbidities (Popa-Wagner et al. 2014; Nucci et al. 2015).

In an *in vivo* study of the middle cerebral artery occlusion (MCAO) model of a rat having type I diabetes shows impaired recovery to the MSC (mesenchymal stem cells) therapy and lack of behavioral improvement when MSC was administered imparting histological difference in BBB (blood-brain barrier) leakage and increase in vascular damage (Chan et al. 2017). Similarly in an another study of the MCAO model of the rat with comorbidity, diabetic rats show greater infarction as compared to non-diabetic rats leading to failure in therapeutics of stroke due to comorbidities (https://www.radiologyinfo.org/en/info.cfm?pg=stroke#:~:text=Because%20treatment%20depends%20on%20the,ultrasound%2C%20echocardiography%20or%20cerebral%20angiography). In SHR strain of rats showed the effect of hypertension

on intracerebral hemorrhage in MCAO model resulted in an improved condition of the brain when implanted BMSC intracerebrally (Chan et al. 2017; Nucci et al. 2015). Analyzing the above-mentioned studies it is concluded that comorbidities associated with stroke increase the risk of neuronal cell damage and interruption of blood vessels resulting in the neurological disorder.

Dosage

In stem cell therapy, one of the major contributing factors is the dosage of stem cells. Various in vitro studies analyzed so far have shown that administration of MNCs at doses higher than 1×105 to -0.25×106 cells/ml leads to a higher risk of arterial occlusion. Other studies suggest that IA administration of BMMCs at a dose of 3×106 cells in MCAO rat models is safe compared to the above-mentioned doses (Popa-Wagner et al. 2014).

In an *in vivo* study conducted on SHR strain of rat testing the efficacy of dose-dependent delivery of MSC administered intracerebral (IC), found a reduction of cerebral blood flow (CBF) signal when a dosage of 1×10^6 and 5×10^5 is given and it resulted into the improved condition of stroke in the rats (Ding et al. 2007) Whereas another comparative study showed when lowering of doses from 2×10^6 to 1×10^6 given in rodent MCAO model they diminished the stroke lesions (Shen et al. 2011). The dose of 2×10^6 causes the administration of stroke lesions to occur frequently, unlikely in the case of dose 1×10^6. Therefore, in their 24 h study post MCAO the animals were IA injected MSCs having a dose of 1×10^5 showing the successful outcome of the stroke reducing infarct volume (Popa-Wagner et al. 2014). Preclinical studies show intravenous (IV) cell dose leads to small infarct volume and initial clinical studies focused that the efficacy of autologous stem cells could be considered safe for delivery at a viable dose.

In a comparison of clinical studies of acute and chronic ischemic stroke, when autologous progenitor and immunoselected CD34+ stem cells were delivered by IC and IA routes at an optimum dosage of 1×10^8 cells, the neurological recovery results were shown to be healthy and neurologically improved (Arora et al. 2012). Whereas the clinical outcome of transplanted modified bone marrow-derived MSC in stroke is given at a dose of 2.5×10^8, 5×10^8, and 10×10^8 cells deposited for 10 min each in 20 μl of cells administered in the peri-infarct zone also show safe and enhanced clinical outcomes (Zhang et al. 2020). Whereas allogenic insertion of fetal neuronal cells IC and IT in a chronic ischemic model of stroke shows the recovery of nerve cells and upper limb functional recovery respectively (Shimbo et al. 2014). The autologous BMMNC when administered in acute ischemic stroke resulted in good recovery when given a dose of $4–6 \times 10^8$; similar is the case study of a clinical trial in sub-acute ischemic stroke when autologous BMMNC given a dose of 2.19×10^8 cells shows IFR in early treatment groups (Zhao et al. 2018). As a result, the best dose for stem cell treatment in clinical trials is yet unknown; typically, a dosage of 1–2 million cells/kg of body weight is proposed (Zhao et al. 2011).

Route of Administration

According to American Stroke Association (ASA), the most effective and safest route of administration defined for preclinical trials is majorly categorized into 4 types (Singh et al. 2020; Chugh 2019): intravenous (IV, administered directly into veins); intra-arterial (IA, administered directly into an artery); intrathecal (IT, administration into the spinal canal, or the subarachnoid space); intracerebral (IC, administration directly into the brain). Among them intracerebral is invasive, shows immune rejection; intra-arterial is invasive and increases the angiogenesis and neuroprotection; intrathecal is mainly for UMSC and improves motor function and lastly, intravenous is less invasive, improves motor function and reduces infarct size (Singh et al. 2020). As per the clinical trials, the IV route is considered to be the most feasible and effective route for stem cell therapy. Delivery routes are defined by a set of preclinical trials based on tolerability, time of intervention, constraint cell production, and the stem cell modes of action (Popa-Wagner et al. 2014; Nucci et al. 2015).

In terms of crossing the BBB, the delivery of BMC cells intravenously is disadvantageous, as they would be stored in the lungs, spleen, or liver (Chen and Li 2020). The same study in which IV cells were deposited in the lungs was found by Fisher et al. 2009 and Hartig et al. 2008 (Zhao et al. 2018). The IA administration has greater advantages to transferring cells to the brain instead of IV and is least invasive than IC administration. In phase I clinical trial, the study of CD34+ cell delivery with the help of IA catheter in the affected MCA when administered within 7–9 days after stroke shows the occlusion of the target vessel or vascular problems. Whereas IC administration of stem cells shows favorable results as it maximizes the dose of cells exactly at the site of time injury close to the location and even circumvent the BBB and hosts immune response (Nucci et al. 2015; Zhao et al. 2018). But in IC administration a slight risk of intracranial bleeding risk in chronic or acute ischemic stroke (Zhao et al. 2011). The study of IC and IV routes of administration in Wistar and SHR strain of rat shows significant improvement in stroke but immune rejection is the only disadvantage faced in IC route and IV route improve the sensorimotor functions the IA route in various studies also shows remarkable improvement with higher graft engraftment and enhanced angiogenesis in rats MCAO models (Ding et al. 2007).

In ischemic cases, the nanoparticle-mediated stem cell administration is normally grouped according to the material composition of nanoparticles, for instance, either by liposomal or polymeric integration. In preclinical trials, liposomes (lipid-based carriers) are referred to as stroke therapeutics. First, for the delivery of hemoglobin, liposomes were used in stroke to restore oxygen in the ischemic neuronal cell via the intravenous (IV) route that penetrates the ischemic heart, sparing the brain's healthy tissue (Moeini et al. 2018). Further research on the models of rats i.e., transient middle cerebral artery occlusion (tMCAO) in which liposomal hemoglobin was integrated after reperfusion through the intra-arterial (IA) path. Several other liposomal formulated drugs, such as FK506 and cyclosporin A, have been given IV to the tMCAO rat model to minimize ischemic stroke oxidative stress (Shen et al. 2011). Liposomal lycopene was intra-gastrointestinally (IG) administered as an

alternative route of delivery in tMCAO (transient MCAO) rats. Liposomal luteolin is given intraperitoneally (IP) resulting in reduced size of infarction and less oxidative stress (Zhao et al. 2011). In the sense of neurological diseases and targeting brain parenchyma toward the target site, the nanoparticle delivery system is a carrier for diagnostics and therapeutics through various routes of administration and can cross the BBB (Zhang et al. 2012).

The intranasal (IN) route leads to functional recovery and decreased neurological deficit scoring by administering liposomal basic fibroblast growth factor (bFGF). Polymer-based carriers such as micelles, dendrimers, nanogels, and others are the most reliable nanoparticles for stem cell administration (Otero-Ortega et al. 2017). Whereas dendrimers constitute an intriguing class of hyperbranched macromolecules with tailored backbone and surface groups have been extensively studied as nanocarriers for gene and drug delivery, via molecular encapsulation or covalent conjugation. Their unique features have led to the creation of a wide range of uses, including the administration of anti-inflammatory medicines. Dendrimers without any drug encapsulated or covalently bound have exhibited therapeutic potential as anti-inflammatory agents. This is mainly due to the immunomodulatory alterations in pathophysiological response of the body's immune system (Avti and Kakkar 2013). For instance, to improve their biocompatibility, the relationship between polymeric nanoparticles and lipid-based stem cell carriers was often administered through the IG route by binding with liposome-coated paraxnotognisenoside outer layer with PLGA (Ferreira et al. 2008). Because of their tightly binding potential and a high degree of stem cell uptake, dendrimers provide great stability and show enhanced functional recovery in ischemic stroke when administered intracerebrally (IC). But a later report documented that dendrimers when administered via the IN route showed improved behavioral changes and greater effectiveness than when administered via IC and IV routes, as they failed to cross BBB. Liposomes are often used in combination with thrombolytic agents such as rTPA, when the IA administration of rTPA with poly-lactic-co-glycolic acid (PLGA) is loaded with antioxidants, catalase, and superoxides reduces the effects of the migration from the subventricular zone (SVZ) of neural progenitor cells results in neurogenesis at the infected site.

Unlike micelles and dendrimers, due to their greater surface area and loading ability, nanogels are efficient and promising nanoparticles for higher beneficial uses. They demonstrate neuroprotective impact in tMCAO rats with the aid of inorganic nanoparticles and with greater ease of crossing BBB. When administered IV with endothelial progenitor stem cells, the superparamagnetic iron oxide nanoparticles (SPIO NPs) coated in silica results in angiogenesis and reduced neurobehavioral disability (Rikhtegar et al. 2019). Neuroprotective results in tMCAO rats with single-walled carbon nanotubes (SWCNTs) directly injecting them into brain ventricles while IC administration with neural progenitor stem cells of hydrophobic multi-walled carbon nanotubes (MWCNTs) improves regeneration at the ischemic core site of stroke, enhancing sensorimotor functions (Sarmah et al. 2018). The IC administration of pigment epithelial-derived factor (PEDF)-enriched exosomes demonstrates increased autophagy and decreased apoptosis. Whereas the loaded

curcumin in the exosome delivery of embryonic stem cells via the IN-route shows reduced inflammation and restores the neurovascular device. IV administration of adipose-derived stem cells (ADSC) resulted in axonal sprouting and glial cell growth with reduced infarct size in the rat endothelin-1 model of stroke exosomes (Wang et al. 2009a). As a result, most studies use IV and IA administration routes because of their least invasiveness, and IG effects of bioavailability are seen in comparison to other path IC routes. Therefore, for this reason, none of them is clinically feasible since IV and IA are more promising ways to administer stem cell therapy mediated by nanomedicine.

Even after different studies on IV, IC, or IA, various limitations have been put forward for the routes in the number of administered cells or their viability and host immune responses. The number of cells delivered via IC injection can be easily quantified but not in the case of IV or IA. Likewise, the bone marrow-derived autologous cells are also unpredictable in the cell no. In terms of clinical studies, at least a two-fold increase in the cell no. for autologous cell delivery has been revealed in various studies. The direct injection of IC is not a precise method of cell administration and results in a poor distribution of cells in the lesion of ischemic stroke (Shen et al. 2011). The major ongoing trials of clinical and preclinical studies are using different routes on the choice of their respective cells and doses postulated in various studies. The best administration route has yet to be found because it is dependent on the delivery time, cells employed, and mechanism of action.

Therapeutic Time Window

However, the therapeutic time window for cell induction in patients where time is a determining factor is more experimental, as is the route of stem cell implantation. Usually, in the ongoing human studies of autologous and allogeneic sources, the time window ranges from a few days to several months post-stroke (Popa-Wagner et al. 2014). Time-based development is known to be the onset of clinical stem cell therapy-induced in the case of stroke with nanomedicine. As we know that rTPA is limited to 3–4.5 h promptly, only the clinically therapeutic time window fulfills important requirements to be taken into account.

To overcome such limitations, nanoparticle-mediated therapy turns out to be a savage one. A timeless example of nanoparticles, i.e., polyethylene glycol (PEG) surface functionalization, i.e., PEGylation, is a classic example of nanoparticles to be regarded as new therapeutics when incorporated with stem cells (Nucci et al. 2015). The efficacy of liposome-mediated hemoglobin in the ischemia model of rat tMCAO shows enhanced effect within 24 h of the time window. Although the multipotent MSC in hemorrhagic stroke with exosome-mediated nanoparticle shows remarkable progress, within 1 day of care, improved white matter and outcomes. Whereas MSC-derived cell membrane vesicles show angiogenesis and reduced infarct size by approx. 7 days after injection of the microvessel with MSC using alpha-smooth muscle action to improve stroke recovery (Ferreira et al. 2008). The integration of exosome ADSC stem cells into endothelin-1 MCAO showed improved neurological functions in axonal sprouting, glial cell growth, and recovery within 15–28 days (Rikhtegar et al. 2019). The therapeutic windows are within 3 days following stroke

if liposomal formulation IV administration is given between 0.5 or 48 hours after reperfusion, both transcellularly and paracellularly. Therefore, nanoparticles are used as an efficient carrier for safe blood stem cell therapy, being non-inflammatory and providing longer circulation duration at the same time showing recovery after therapeutics with a short period.

In a study conducted on the MCAO model of a rat, the transplantation of NSCs after 48 h of stroke leads to better survival instead of transplantation after 6 weeks of stroke. In the above comparative study, the transplantation of MSCs after 2–3 weeks of ischemia shows enhanced regeneration ability including neurogenesis and angiogenesis (Zhao et al. 2018). Whereas the implantation of cells at an early stage (within hours to 1–2 day of stroke) is advantageous as it allows the potential delivery of cells into the periphery of the brain to suppress the leaky BBB flows; producing inflammatory chemokines that arose due to stroke may act as chemotactic cell and the induced stem cells may reduce the stroke inflammation in the brain (Sinden et al. 2017).

The majority of preclinical studies have been conducted within 3 days after stroke via bone marrow or hematopoietic stem cells. This therapeutic window offers enhanced potential of NSCs implantation post-stroke and has shown greater recovery and efficacy in about one week (sub-acute phase) and greater than 3 weeks (chronic). The chosen time window of 24 h post-stroke for the administration of BMSC in aged Wistar rats resulted in improved conditions of occluded rats (Shen et al. 2011). Also, the prescribed time is 2 days post-stroke in Sprague-Dawley male rats show improved neuroprotection and angiogenesis (Tatarishvili et al. 2014). Whereas in acute and sub-acute ischemic stroke clinical trials using BMNC time window of 1–3 days of USA patients, 5–9 days of Spain patients and Fetal neural cells administered after 4 months in Russia patients showed the improved result of angiogenesis, synaptogenesis, rescuing of blood flow respectively (Shimbo et al. 2014). The prescribed time window for cell transplantation usually depends on the type of stem cell used and thus the pathway of action. A better understanding of the cellular interaction with neuronal cells can be used to optimize the optimum time for cell implantation on the brain of the patient by patient.

7.3.2.2 Mechanism of Action of Stem Cell

Stem Cell Homing, Tracking, and Survival

Homing is the phenomenon where the cell will migrate toward the organ of its origin via the help of various chemokines and receptors. Stem cell homing is an endogenous multistep process that is usually used by the exogenously administered stem cells likewise HPSCs, MSCs, NSCs, etc. Homing is of great importance for the aim of persuading stem cells at the injured site (Zhao et al. 2018). Generally, the ability to locate and adapt in an environmental niche of the circulating stem cells or the exogenously administered cells is called stem cell homing. Hence, the process of homing is quite useful in transplantation, regenerative therapies, or the migration of cells to their origin. The study of *in vitro* homing used to be done by using a chamber or trans-well system to analyze the migration and chemotactic movement of cells.

But *in vivo* studies include tracking, imaging, and surviving off the homing cells to maintain the check on homing efficacy. When it comes to cerebral ischemia, the IV injection in rats leads to the deposition of cells in the lungs, liver, or spleen; which later results in the micro-occlusion of the vessels and less efficacy. To improve and enhance the homing efficacy the cell therapy can be altered in culture, cell sorting, and modifying cell surfaces (Kwon et al. 2019).

Meanwhile, a lot of factors affect the survival of cells in cerebral ischemia (acute phase) likewise less blood supply, decline of tropic factor, inflammatory and immune responses, hypoxia, etc. The survival of homing genes can be promoted by modifying genes with several factors like Bcl-2 and SCF (stem cell factor) which increase the chances of survivability of ESCs and MSCs. In an animal model of stroke, the survival of NSCs increases due to the overexpression of several growth factor genes like VEGF, GDNF, BDNF, and Akt-1. To track the fate of stem cells, their migration after transplantation in *in vivo*; various techniques for cell labeling were used for instance magneto dendrimers, fluorescent proteins, Feridex (iron-containing agents), Gd-DTPA (gadolinium-diethylene triamine pentaacetic acid, i.e., paramagnetic particle, etc.). But along with advantages, there are few limitations of the labeling which are quite toxic and harmful to proceed with cell signaling. Other cell labeling techniques include 99-technetium SPECT, 111-indium SPECT, or 18-fluoro deoxyglucose positron emission tomography (FDG-PET) where the radioisotope labels in combination with MRI (magnetic resonance imaging) (Samarasinghe et al. 2012).

The efficacy of cell labeling is directly proportional to the cell dose, whereas the viability of the cell is low with indium labeling and has a long incubation time. But the major concern in cell tracking is that these labels may interfere with the viability of cells with normal cell function. It is demonstrated that NSCs were labeled with MRI to help in visualizing the migration of NSCs in rats at the injury site. Alternatively, put forward several other non-iron MRI techniques of fluorine-labeled cells visualized with MR spectroscopy also act as an additional value in cell therapies (Bain et al. 1995). Cell-based therapeutics is considered to be the most advanced field in medicines which collaborate with a range of other fields likewise transplantation, biomaterial, and stem cells. Nanomedicine provides a potential and alternative strategy which is applied in various neurological disorders. It is used for the targeted drug/cells to get delivered, replace or repair cells and for diagnostic imaging purposes. For instance, MSCs have a unique ability to migrate toward the damaged nerve site and secrete various types of secretome to induce regeneration and repair of nerves (Avti and Kakkar 2013). The non-genetically modified MSCs are assisted via nanoparticles because of their low immunotoxicity in comparison with the genetically modified ones. In nanoparticles assisted stem cell therapy showcasing their homing and apoptotic ways, the expression of MSCs was enhanced by adjusting them with the membrane conjugated nanoparticles in post nerve regenerative therapy. Internalization of MSCs loaded with nanoparticles will greatly enhance the delivery of the cells in nerve regeneration (Zhang et al. 2001). A major pathological cause of stroke is inflammation which leads to damage of peripheral innate response and the disruption of the blood-brain barrier, which is usually triggered by the

release of cytokines and various other inflammatory factors. The use of nanoparticles acts as potential therapeutics for the delivery of stem cells into the injured area. The nanoparticles are the colloidal carriers of natural and synthetic origin that vary in size from 1 to 1000 nm (Reubinoff et al. 2001). Naturally, they are composed of proteins, polysaccharides, or chitosan, whereas the synthetic ones are made up of polymers like polylactic co-glycolic acid (PLGA), or inorganic agents like gold, silica, or alumina. Various modified nanoscale surfaces are designed and aligned in a variety of stem cells, for example, MSCs, ESCs, HPSCs, or NSCs. Park et al. in a study conducted that the effect of TiO_2 nanotubes surface on the MSCs of rats and find out that 15–30 nm of spacing provides an optimum scale for clustering of integrins, cell proliferation, differentiation, and migration which results in the cellular behavior of rat to adhere, spread and grow leading to the severe impairment of nanotubes more than 50 nm size and showed apoptosis of cells at a level of 100 nm size of nanotubes (Ying et al. 2003). Usually, the synthesis of nanoparticles for regenerative procedures mainly focuses on the entrapment and delivery of the cells for therapeutics. The most promising nanoparticles for stem cell therapy are magnetic nonmaterial, i.e., iron oxide nanoparticles which are tending to bind the cell membrane externally or are inserted into the cytoplasm (Avti et al. 2013b). Due to the magnetic properties of NPs, the labeled MSCs or ESCs can be easily tracked over a period of time using imaging modalities like MRI or PEM, which reduces the repeated dose of the contrast agent *in vivo* (Singh et al. 2019; Wei et al. 2005). Various other nanoparticles are used for the stem, cell labeling, and *in vivo* tracking including quantum dots, carbon nanotubes, and polyplexes for the intracellular delivery of genes and proteins. The scaffold of the nanometer-scale is used for the transplantation of stem cells and their differentiation. Usually, labeling procedures use two basic approaches: either they attach magnetic nanoparticles to the surface of stem cells or internalize them via endocytosis and phagocytosis (Wei et al. 2005; Yanagisawa et al. 2006). For instance, the SPIO (supermaganetic iron oxide) were internalized via HMSC at a concentration of 23.4 pg iron per cell without any transfection agent (Ferreira et al. 2016). Whereas nanofibers assemble scaffold for tissue engineering of stem cells were implanted on 3D scaffold formed by nanofibers and hence the cell proliferation and differentiation are considered after the culture of stem cell or the 3D scaffold for final implantation (Tae-Hoon and Yoon-Seok 2012). Nanotechnology-regulated stem cell therapeutics is a novel approach in the biomedical field to enhance the regeneration ability of damaged neurons in various neurological disorders. Therefore, nanoparticles like quantum dots, carbon nanotubes, and magnetic nanoparticles are used in different ways for imaging or tracking, gene/drug in delivery, and for the proliferation and differentiation of stem cells (Solter 2006).

Pathway of Action

Stem cell therapeutics for stroke have enhanced the applicative means of repairing damaged brain, neuroprotection, neuroregeneration, and angiogenesis in the affected areas. The capacity to differentiate into different types of stem cells is the remarkable property of stem cells for various neurological disorders (Reubinoff et al. 2000). The endogenous repair process is stimulated by the administration of stem cells

exogenously but is unable to replace cerebral tissue. In a study, it is postulated that intravenously administered cells home into the injured sites hence replacing the apoptotic neurons. But as per the current conducted studies, it is analyzed that stem cells release many trophic factors likewise VEGF, BDNF, IGF, and TGF which stimulate brain plasticity and repair mechanism (Thomson et al. 1998; Hao et al. 2014). The intravenous stem cells work as 'chaperones' by upregulating the growth factors, inhibiting apoptosis, and enhancing synaptogenesis. The treatment strategies for stroke via stem cells translational and basic researches showing regenerative potential via various types of stem cells are ESC (embryonic stem cells), iPSC (induced pluripotent stem cells), MSC (mesenchymal stem cells), and NSC (neural stem cells) (Zhang et al. 2001). Refer Fig. 7.4.

ESCs are the pluripotent stem cells derived from the inner cell mass of the blastocyst; having the ability of self-renewal and differentiation into vivid types of cells (Reubinoff et al. 2001; Ying et al. 2003; Wei et al. 2005). Also, the major advantage of ESCs is their capability of unlimited expansion and induces the differentiation into neural lineages to meet the requirements of cells *in vitro* (Yanagisawa et al. 2006; Tae-Hoon and Yoon-Seok 2012; Solter 2006). In severe focal ischemia when the ESCs have transplanted into the rat cortex the cell surface markers of neurons, oligodendrocytes, astrocytes, and endothelial cells were found in the lesion cavities which are derived by ESCs (Reubinoff et al. 2000; Thomson et al. 1998; Hao et al. 2014). Whereas in another study the intra-arterial transplantation of the ESCs derived neuron-like cells enhance the function of dopaminergic activity and hence recovers the dysfunction of focal ischemia in rats via the MCAO model. The transplantation of human ESCs (from endothelial cells and mural cells)

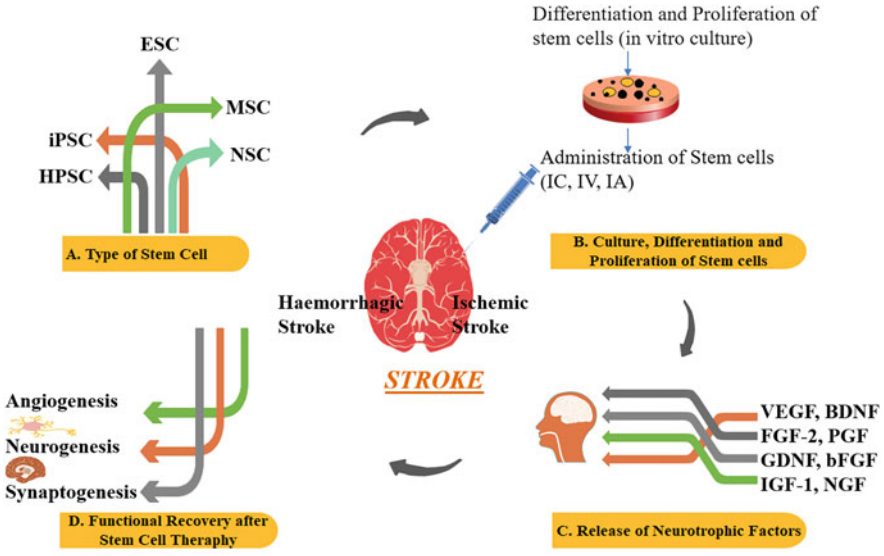

Fig. 7.4 Mechanism of action of stem cells

intra-arterially not only increases the cerebral blood vessel and vascular density in the striatum of ischemia but also reduces the infarct size and inhibits apoptosis enhancing the neurological recovery in mice MCAO model (Daadi et al. 2008; Kim et al. 2007).

While the intracerebral transplantation of mouse ESCs not only improves the motor and sensory function of the rat but also reduces the infarct size. The major disadvantages of ESCs were their malignant transformation and sometimes the formation of teratoma. Otherwise, the ESCs transplantation provides potential therapeutics avoiding malignant transformation when implanted *in vivo* (Hicks et al. 2009). In various studies, it is explored that the effective way of treating stroke via ESC-derived neural stem cells/progenitor stem cells (NPSCs) showed improved behavioral response, reduction in the infarct area, and enhanced number of neurons. Despite the fact of having different routes of administration, animal models, and transplantation cell sources, the stem cells help in aiding the neurological impairments in stroke treatment (Bühnemann et al. 2006; Hayashi et al. 2006).

IPSCs are pluripotent stem cells generated directly from the somatic cells. They are quite similar to the naturally occurring pluripotent stem cells, i.e., ESCs in various cellular biological properties (like stem cell markers, stem cell genes, telomerase activity, growth properties, and morphologically) (Takahashi and Yamanaka 2006). The iPSCs differentiate similarly as ESCs into a fully differentiated tissue likewise neural differentiation into different neurons (Cai et al. 2010; Tat et al. 2010). The only major disadvantage of using iPSCs is the formation of teratoma similar to ESCs. Along with that, the beneficial impact of iPSCs is that because they can be directly derived from adult tissue, they not even bypass the embryo, but can be formed in a patient-matched form (means that each individual can have their pluripotent cell line) due to which unlimited supply of autologous iPSCs can be transplanted without being rejected from the immune system (Chen et al. 2010; Oh et al. 2020; Kawai et al. 2010). The therapeutic effect of iPSCs subdural transplantation results in the effective reduction in infarct volume and potentially improves the rat's behavior in the MCAO model while preferring rotarod and grasping tasks. In another study, the transplantation of iPSC via adult human fibroblasts in the MCAO rat model migrates toward the injured site and effectively improved the sensorimotor functions (Martínez-Garza et al. 2016; Hankey 1999). So, it is concluded that iPSCs are considered as a promising approach for therapeutics in stroke only if the tumorigenesis is being controlled in the iPSCs cells (Gore et al. 2011; Yamashita et al. 2011). MSCs or mesenchymal stromal cells or medical signaling cells are the multipotent stem cells that can further differentiate into a variety of other cell types like osteoblasts, chondrocytes, myocytes, and adipocytes (Reis et al. 2017; Chia et al. 2020). MSCs are comprised of non-HSCs in the bone marrow, and by the Mesenchymal and Tissue Stem Cells Committee of International Society for Cellular Therapy, there were three criteria proposed to identify MSCs, i.e., the plastic adherence of isolated MSCs in culture, more than 95% of culture show the expression of CD105, CD73, and CD90 and absence of various expression markers CD34, CD45, CD14 or CD11b, CD79a, or CD19 and HLA-DR in more than 95% of culture (Li et al. 2002). MSCs have the ability to self-renewal and

differentiate into neural cells when transplanted *in vitro* analyzed by the expression of neural markers NeuN which migrate to the site of brain lesions. The modification of genes with exogenous cytokines, i.e., GDNF, BDNF, FGF-2, PGF, and angiopoietin-1 showed the effective results of MSCs in treating stroke (Chopp et al. 2008; Friedenstein et al. 1966). The MSCs were transplanted IV or IC which could migrate toward the injured brain site and shows improvement *in vivo* studies of stroke. The migration of MSCs is mediated by the increased chemokines, i.e., SDF-1 in nearby surroundings and CXCR4 presenting on MSCs (Zou et al. 2010; Dominici et al. 2006). Various other factors like IL-8, MCP-1, MIP-1a, and VEGF are also involved in the process of migrating MSCs to the injured site of the brain. Whereas the IV route of MSCs is superior instead of IC injection due to its less invasiveness and more extensive neuroprotection (Pittenger et al. 1999; Kurozumi et al. 2004a). Several experimental ischemic stroke models showed the mechanistic behavior of MSCs for instance, by the neural differentiation into neurons or glial cells; increase in neurotrophic factors (SDF-1, GDNF, BDNF), increase angiogenesis in rMCAO; or by decrease apoptosis and differentiation of NeuN, GFAP in rMCAO model via BDNF-modified hBMSC; or by decreasing the infarct area by IV transplantation of MSC in rMCAO increase angiogenesis, homing and decrease apoptosis, inflammation, and oxidation (Kurozumi et al. 2004a; Liu et al. 2006). The secretion of trophic factors by MSCs is the contributing factor in stroke treatment. Likewise, BDNF is constitutively expressed by MSCs which significantly increases the therapeutic effect of MSCs. Several other neurotrophic factors HGF, VEGF, NGF, bFGF, FGF-2, and IGF-1 implicate the endogenous repair mechanism via MSCs (Toyama et al. 2009). The trophic factors like neuroprotection, angiogenesis, synaptogenesis, neurogenesis, inflammatory, and immune-modulatory response play a crucial role in MSC therapy in stroke (Yoo et al. 2013). The neuroprotective effect of various neurotrophic factors, i.e., SDF-1, VEGF, GDNF, BDNF, ICF in several studies is found to be quite effective in treating ischemic animal brain (White et al. 2000; Ikeda et al. 2005). The stimulation of these factors can be directly regulated by MSCs or indirectly by the host cells. The neuroprotective effect is mediated via these factors majorly including increased nerve cell survival, decreased excitotoxicity of glutamate, inhibition of apoptosis, and anti-inflammatory action. According to a recent finding on a rat model of stroke the MSCs increase the tPA activation which enhances the axonal and synaptophysin production which results in recovery of stroke (Ding et al. 2007; Chen et al. 2001). Angiogenesis increases the expression of ß-1 integrin and other trophic factors secreted via MSCs, i.e., VEGF, BDNF, IGF-1, bFGF, GDNF, and TGF enhance the production of new blood vessels and synaptogenesis (formation of synapse between neurons in the nervous system). Another mode of action by which the MSCs repair the neurological functions in ischemic stroke is the increased endogenous neurogenesis which attributes to increased angiogenesis and improvement in CBF (Shen et al. 2011; Xin et al. 2010). Modulation of inflammatory and immune response by MSCs in ischemic stroke is an effective mechanism of neuroprotection in stroke. When Ad-MSCs have injected IV in rMCAO the IL-18, TLR-A, and plasminogen activator inhibitor (PAI-1) of mRNA expression reduce the inflammation and brain area. MSCs not

only decrease the systemic inflammatory cytokine levels (IL-6, TNF-beta, IF-gamma, and MCP-1) expression but also secrete TGF-beta1 to suppress the immune propagation in rat's brain having an ischemic stroke (Leu et al. 2010; Li et al. 2002).

NSCs are the self-renewal multipotent stem cells that firstly lead to the formation of radial glial progenitor cells which further generate the neurons and glia of the nervous system. NSCs generate the neurons and glia of the nervous system (Ming and Song 2011; Thored et al. 2006). NSCs will primarily differentiate into various cells like neurons, astrocytes, and oligodendrocytes. The specialized NSCs in the adult mammalian brain are present in the subgranular zone in the hippocampus dentate gyrus, the subventricular zone around lateral ventricles, and the hypothalamus (Kim 1996; Zhang et al. 2002). NSCs are normally of two types, i.e., endogenous NSCs and exogenous NSCs. The endogenous NSCs usually function by producing various neurotrophic factors like NGF, GDNF, and regulated the inflammatory-immune response, promoting proangiogenic complexes, i.e., netrin-4, laminin, integrin, and various types of secreting factors that promote synaptogenesis, i.e., thrombospondins (Otto et al. 2002; Hallbergson et al. 2003). Different approaches were aimed at promoting the endogenous NSCs by enhancing the survival, proliferation, and differentiation into other types of cells. For instance, increased concentration of cytokines (BDNF and VEGF) by modifying genes in the local area of brain promotes the malignant activity of endogenous NSCs towards the injured site of the brain (Wang et al. 2009b; Chou et al. 2006).

Erythropoietin also increases the process of neurogenesis by the cytokines (BDNF and VEGF) in the rat MCAO model. Whereas exogenous NSCs are obtained from ESCs, iPSCs, bone marrow, Ad-MSCs, and E-NSCs; exogenous stem cells will proliferate *in vitro* by stimulating them through various growth factors, i.e., EGF, FGF, LIF (leukemia inhibiting factor) which remarkably differentiate into neurons, astrocytes, oligodendrocytes, etc. in rat MCAO model of ischemic stroke, the E-NSCs surviving rate is quite higher than that of adult-NSC, making the E-NSCs more efficient in reducing infarct size and improve neurological functions (Plane et al. 2008). In a study, it is postulated that human fetal NSCs are less tumorigenesis instead of ESCs and that is why fetal NSCs tend to show a strong capacity of proliferation *in vitro* and differentiate into neurons *in vivo*. In response to various chemo-attractant stimuli via rolling and adhering to endothelial and their transmigration leads to circulation and migration of them to the injured site of the brain (Sims et al. 2009). The communication between stem cells and endothelium is mediated via the VCAM-1 and integrins alpha-2, alpha-6, beta-1. Also, the guidance given to NSCs to reach the injured targeted brain site is due to various chemokines such as SDF-1, MCP-1, ANG-1, and slit. IC transplantation of NSCs comes out to be the most advantageous route that depends on the high number of grafted cells, stabilization of BBB, and reduces the ROS while maintaining the reperfusion (Coelho and Giraldi-Guimarães 2014). To obtain a better distribution of NSCs to the injured area and also to avoid invasive surgery IV route of NSCs delivery is better as compared to IC. The underlying mechanism of NSCs in the recovery of ischemic stroke is still unclear, although cell replacement is considered to be the

recognized mechanism of transplanted NSCs. In various animal studies usually, NSCs will be differentiated into neural or glial cells (Zhang et al. 2013). In terms of neuroprotection effect the number of NSCs survival in the infarct area was too less to replace the damaged/lost neurons. Though the neuroprotection is offered by various cytokines secreted by exogenous NSCs likewise VEGF, BDNF and neurotrophins result in functional recovery after ischemic stroke by enhancing angiogenesis, immunomodulation, and neurogenesis (Gao et al. 2018; Barzegar et al. 2019). The NSCs when injected into the cortical infarct cavity of the rat stimulate the neurogenesis in the SVZ ipsilateral area of stroke. Modulation of inflammatory and immune response is mainly regulated by the microglia; which is considered to be the main inflammatory regulator in focal stroke brain tissue (Shen et al. 2007; Ding et al. 2017). The IV administration of NSCs leads to the reduction of OX-42+ microglia and infiltration of neutrophil in the brain infarct area resulting in the attenuation of cerebral and splenic activators of TNF-α, IL-6, and NF-kB which promotes the neuroprotection in the rMCAO model. Also, the enhanced formation of new brain microvessels in the brain imparts functional recovery in ischemic stroke (Chen et al. 2011; Choi et al. 2016).

Cerebral ischemia leads to neuronal death due to various pathophysiological causes. The TPA or mechanical thrombectomy as a treatment for ischemic stroke is usually performed as a therapeutic procedure to restore blood supply in the brain. In general, several neuroprotectants have been designed to improve reperfusion-induced damage, but none of them have been clinically approved to date. Due to BBB, shorter drug distribution duration, safety, and toxicity, etc., this failure may be due to inefficient delivery of drugs into the brain. But recent developments in nanotechnology emerge with the aid of nanoparticles as a new way to treat stroke. Nanoparticles act as cargo vehicles to, deliver drugs and make it possible to cross BBB, raising the probability of drug accumulation at injured sites (Zhang et al. 2018). By supplying neuroprotective agents, siRNAs, and inflammatory drugs to increase the cure for stroke injuries, nanomedicine provides great possibilities in stroke therapy. In general, nanoparticles with a size of 50–200 nm have been developed and help to encapsulate drugs to reach the target site. As a stroke therapeutic agent, nanoparticles are ideal carriers because of their smaller size than a cell and larger than a molecule.

The formulations of theranostics are a new and promising approach of combining stroke diagnostics and therapeutics. A novel platform will enhance the outcome in the treatment of stroke patients, mediated by nanomedicine along with stem cell therapeutics. Due to their non-toxic, biocompatible, biodegradable, stability in blood, diameter less than 100 nm, being anti-inflammatory, crossing the BBB easily, and having prolonged circulation time NPs are feasible in facilitating the stem cells as effective therapeutics against stroke (Fernandes et al. 2018). Various NPs in use for theranostic means are polymeric NPs, iron oxide NPs, carbon material NPs, and hydrogel nano scaffolds (Panagiotou and Saha 2015).

For rapid identification in methods of diagnosis such as CT and MRI scans nanoparticles have been used. According to the nanotechnology point of view, we used to design the nanoscale material in a size of 50–200 nm and can be used to

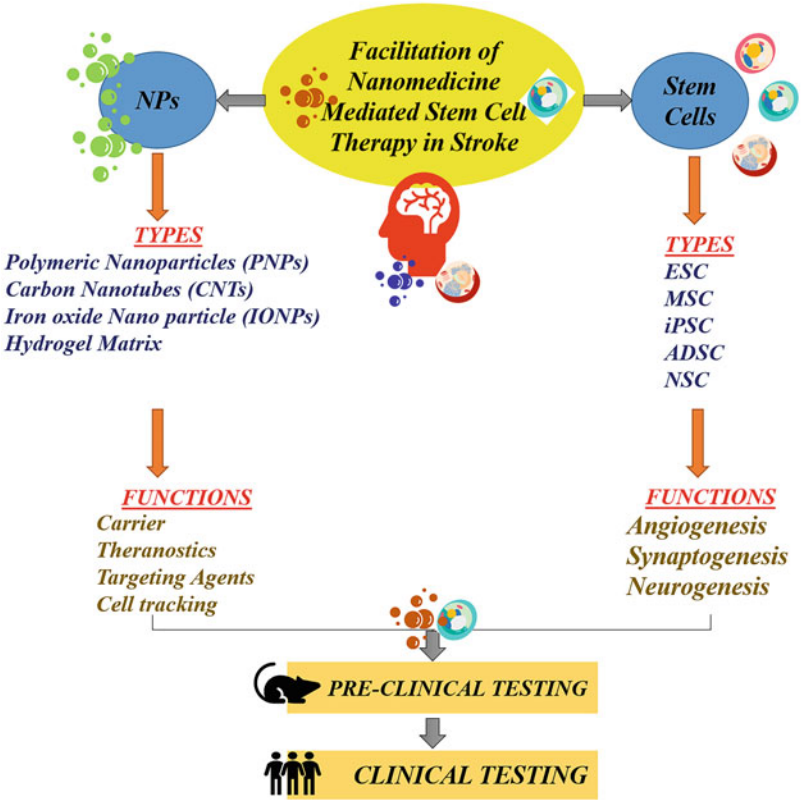

Fig. 7.5 Facilitation of nanomedicine-mediated stem cell therapy in stroke. *NP* nanoparticle, *ESC* embryonic stem cell, *MSC* mesenchymal stem cell, *iPSC* induced pluripotent stem cell, *ADSC* adipose-derived stem cell

improve the therapeutics of stroke with stem cells. NPs are not only limited to stem cell therapy delivery rather than are used in the delivery of drugs, neuroprotectants, and anti-inflammatory agents via PLGA NPs, chistan NPs, or PAMAM dendrimer (Wong et al. 2013). On the other hand, while facilitating the role of nanomedicine mediated stem cell therapy major benefits includes the preparation of substrate for amplifying stem cells on a larger scale to repair and regenerated neurons in neurodegenerative diseases. Refer Fig. 7.5.

To achieve successful transplantation of stem cells in stroke and to overcome the obstacles, the unique features of nanoparticles such as high surface to volume ratio, distinct mechanical, physical, thermal, electrical, magnetic, and optical characters make them ideal to overcome the barrier in neural stem cell therapy. Nanotechnology-based approaches are recently being investigated for a wide range of biomedical fields due to their potential biostability, biocompatibility, and biodistribution (Avti et al. 2011, 2012). The creation of nanocarriers for the effective transport of therapeutic agents to the particular position has taken a lot of time and effort. The

macromolecule-based nanocarriers utilized for this purpose should be non-cytotoxic, maintain their integrity before reaching the target location, and improve the product's efficacy (Avti et al. 2013a). The NPs can be modified into desired size, shape, composition, and properties (Mc Carthy et al. 2015), for instance, metallic nanoparticles iron oxide NPs (IONPs) which possess supermagnetic properties, i.e., supermagnetic iron oxide (SPIO NPs) are biocompatible, biodegradable, and constituting inert iron oxide core, majorly of magnetite and a coating layer surface of the functional group makes them a promising tool for regenerative therapy in neurodegenerative disease. The supermagnetic property of SPIO NPs enables them to migrate toward the injured area (Abdal Dayem et al. 2018). For instance, the Ferucarbotran SPIO NPs efficiently promote the differentiation and proliferation of hMSCs by counteracting with the intracellular H_2O_2 resulting in the improvement of the cell cycle progression by upregulating the cell cycle-related proteins likewise cyclin D1, Cyclin B, and cyclin-dependent kinase 4. Therefore, it is estimated from this analysis that the internalization of hMSCs into the SPIO NPs by examining the cellular effect of ferucarbotran labeling depicts that the magnetic NPs possess intrinsic peroxidase activity and when administered inside the SPIO particle are transferred to lysosomes where they are being degraded; releasing free iron into cytoplasm because iron depletion arrest cell cycle and cause apoptosis. The released iron plays an important role in cell cycle proliferation, progression and inhibits apoptosis; promoting the MSC level at the injured ischemic site (Huang et al. 2009).

The MRI-guided localization and transplantation of SPIO NPs-labeled ADSCs at the infarct region of the brain help in appropriate diagnosis. The ADSC harvested from mice were labeled with SPIO ferumoxide particles using poly-L-lysine. The ADSC viability, proliferation rate, and iron release were measured after the SPIO labeling, and sensitivity of MRI detection with labeled ADSC ex vivo. After 2 weeks of MCAO, the MRI was performed to guide the stereotactic transplantation of SPIO labeled ADSC into brain tissue adjacent to the infarct area. Their observation after sacrifice concluded that ADSC was efficiently labeled with SPIO without any loss of cell viability and enhanced proliferation. Hence SPIO NPs labeled ADSC proved useful in the precise transplantation and proliferation of stem cells at the infarct site of ischemia (Rice et al. 2007). The enhanced magnetic properties of SPIO NPs under the influence of external magnetic field help in image-guided transplantation at the accurate infarct region, thus making it a promising approach (Nucci et al. 2015).

Carbon nanotubes (CNTs) are tubular, small, hollow cylinder tubes made of carbon atoms with a nanometric diameter, making them high in mechanical power, stability, thermal and electrical conductivity. CNTs have been internalized as a scaffold, despite their peculiar physiochemical properties, resulting in progressive cell attachment, differentiation, and proliferation (Avti et al. 2013c; Fernandes et al. 2018). For the very first time, CNTs were used to analyze the neuroprotective function of SWCNTs in the rat MCAO model via intraventricular administration of amine-modified SWCNTs. The resulting outcome was neuroprotection, improved neurobehavioral rehabilitation, and Akt pathway inhibition; the neuroprotective function of SWCNTs was inferred. In addition, an *in vivo* study of an induced ischemic model of stroke showed that this nanomaterial was effective when

impregnated with progenitor cells of neurons. The impregnation of hydrophobic CNTs with SVZ NPCs exhibits improved neurobehavioral, reduced infarct volume as compared to the control group. The maximum HPCNT-SVZ NPCs gathered near the ischemic site lead to the repairing and differentiation of SVZ NPCs into mature neurons with increased angiogenesis, synaptogenesis, and decreased apoptosis (Moon et al. 2012b).

The CNTs can be conjugated with other molecules such as polymers, proteins, nucleic acids and can play a significant role as a sensor, biomarkers, and drug carriers in therapeutics of neurogenerative disorders. For instance, the recovery of endothelin-1-induced stroke model via the functionalized-CNTs (f-CNTs) shows improved results. Here the silencing of caspase-3 using CNTs mediated SiRNA *in vivo* stroke model. The stereotactic administration of caspase-3 SiRNA (SiCas-3) delivered via f-CNTs leads to the reduction in degradation of neurons and inhibition of apoptosis, promoting functional recovery in sensorimotor functions of the rat. Thus, concluding that the *in vivo* gene silencing of Cas-3 mediated by f-CNTs: SiRNA causes neuroprotection against ischemic stroke (Al-Jamal et al. 2011).

Another carbon-based nanomaterial are fullerenes, which are hollow spheres of carbon atoms having zero dimensions, are used in stroke therapy as they can cross BBB and when hydroxylated they act as a neuroprotective agent by scavenging the free radicals. As a scavenger of free radicals, fullerenes not only help but also efficiently minimize the excitotoxicity of glutamate and avoid neuronal cell death (Fernandes et al. 2018). A derivative of fullerene, i.e., Fullernol (OH-F), is the radical scavenger, when conjugates with glucosamine (GlcN-F) and administered IV in normotensive Wistar rats and hypertensive SHR rats spontaneously after tMCAO. After administration, they were analyzed via MRI showing significant improvement in neurobehavior and reduction of infarct volume in both Wistar rat and SHR rat groups. Also, neuronal loss is prevented in the perilesional area, concluding the reduction of cellular damage and inflammation in stroke therapeutics mediated by Fullerene carbon nanomaterial (Fluri et al. 2015).

A sub-type of carbon nanomaterial, graphene (G) is an atomic thick layer of carbon atom arranged in the 2D honeycomb structure. The coated substrates of graphene and graphene oxide (GO) are biocompatible, biodegradable, helping to allow iPSCs to bind to cells, proliferate and support the surface for further differentiation, giving strong bioactivity (Fernandes et al. 2018; Li et al. 2011). VEGF-loaded IR800 conjugated GO was used as a theranostic agents for multimodal imaging – monitored targeting therapeutics increased angiogenesis of ischemic muscles. The GO-IR800-VEGF upregulates the VEGF receptors and elevated the level of growth factors for a prolonged period of time *in vivo* by IV administration, suggesting GO as a theranostic agent for treating ischemic stroke (Sun et al. 2013).

Polymeric NPs consist of different types of polymers, commonly used for the manufacture of nanocapsules and nanospheres. Polymers are repetitive subunit macromolecules and play a major role in biological life (Panagiotou and Saha 2015). The PNPs are primarily divided into two subgroups, i.e., natural and synthetic PNPs. The synthetic PNPs are polycaprolactones (PCL), poly lactic-co-glycolic acid (PLGA), and polylactide (PLA), while the natural PNPs are chitosan, collagen,

nucleic acid, retinoic acid, hyaluronic acid, and alginate (Wong et al. 2013). Due of their excellent physiochemical properties, such as biocompatibility and low immune reactivity, PNPs are commonly used in stem cell therapy. PNPs are used for the encapsulation or immobilization of bioactive factors within stem cells to prevent enzyme degradation. Being cationic, they interact via the electrostatic force to form a particle of about 100 nm in diameter with the negatively charged nucleic acids or the growth factors (Boisserand et al. 2016). For example, polymeric NPs loaded with retinoic acid increased vascular control of NSC proliferation, differentiation, and post-ischemic stroke survival. Retinoic acid-nanoparticles (RA-NP) created a favorable pro-angiogenic environment by enhancing the growth factors like VEGF, GDNF, BDNF, etc., to ensure safe and efficient repair of neurons in the ipsilateral region. This study actually emphasis that the RA-NP increased the endothelial cell proliferation up to 83 folds in comparison to free RA, increased tubular network formation, reduced apoptosis against ischemic stroke. It is therefore assumed that RA-NP protected the endothelial progenitor cells from ischemic apoptosis, stimulated the release of neuronal restitution growth factors, and increased the amount of NSCs at the injured ischemia location (Ferreira et al. 2016).

In an other study, the single injection of ONO-1300-loaded PLGA nanosphere decreased the impact of ischemia on the brain in the MCAO subjected rats. When they are encapsulated in the PLGA nanosphere, subcutaneous administration of ONO-1300 to the ischemic brain is enhanced and helps to precisely migrate the stem cells at the injured site of the brain, serving as a nanocarrier in the brain stroke treatment (Hazekawa et al. 2012).

The hydrogels are polymer networks that contain a large amount of water and often have extracellular matrix in their structure (ECM). Hydrogel can use self-assembling peptides (SPAs) to construct a nanofibrous structure that mimics ECM. ECM can be combined with GDNF, BDNF, VEGF, etc., additive neurotrophic factors to improvise the neural stem cell transplantation setting (Panagiotou and Saha 2015; Zhong et al. 2010). The direct transplantation of the neural stem cell at the ischemic site has many limitations, such as the massive death of transplanted neural stem cells due to immune response or divergence of the stem cell from the injured site to other nearby tissues. To overcome these limitations, a unique biopolymer hydrogel matrix is developed to implant the neural stem cells in a favorable environment. Thus, in both *in vitro* and *in vivo* settings, the hydrogel matrix composed of cross-linked hyaluronan and heparin sulfate resulted in a substantial promotion of neuronal progenitor cell line survival. Therefore, the hydrogel matrix facilitates the survival of stem cells, decreases cell tension in the infarct cavity, and provides translational stroke rehabilitation therapy (Zhong et al. 2010).

In contrast, human NSCs based Matrigel scaffold transplantation improves the behavioural outcome of focal cerebral ischemia following post-ischemic treatment in rats. Together with Matrigel scaffold, cultured neural stem cells were transplanted to demonstrate decreased infarct volume, survival, and neuronal differentiation at the injured brain site (Jin et al. 2010). Another research postulated that the delivery of iPSCs-NPCs inside a hyaluronic acid matrix to the stroke infarct cavity improves the differentiation of transplanted cells and helps to reduce ischemia (Lam et al. 2014).

After an ischemic event, biomaterial scaffold has a great potential to treat stroke. Linking nanotechnology and stem cell therapy will also help the field of regenerative medicine and promotes the combination of nanotechnology and stem cell therapy in the ischemic stroke therapy.

7.4 Summary and Future Prospective

Stroke remains a main cause of mortality and morbidity globally; therefore, in the coming years, there is an utmost need for greater translational studies to overcome the challenges. Nanoparticles' potential to mediate stem cell proliferation, migration, and differentiation may help in neuroregeneration, and they constitute an increasingly flexible platform for stroke therapy. Therefore, nanoparticle-mediated stem cell regeneration is considered an effective therapeutic strategy against stroke. According to Clinical trial government data (www.Clinicaltrials.gov) approx. 2045 clinical trials are on track for stem cell therapy. Various effective ways are being developed to prevent stroke, for example, by delivering stem cells via nanoparticles or other biomolecules to the cytosol or nucleus. Theranostics of nanoparticles regulating stem cell response can help reduce infarct volume when given at the right dose taking into consideration the patient's age, sex, and other concomitant conditions thereby the neurological deficits in stroke patients can be improved.

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The Influence of Preconditioning on the Homing Behavior of Stem Cells

8

Mohammad Tayyab Shamsh, Santosh Kumar Yadav, Nida, Fatima Zaidi, Abdul Quaiyoom Khan, and Syed Shadab Raza

Abstract

Stroke is the second leading cause of disability and death in the world. Therefore, there is an urgent need to develop treatment strategies for stroke. Stem cell therapeutics appears to be a promising alternative to approved thrombolytic or thrombectomy approaches. Stem cells can repopulate into various cell types in an ischemic brain by stimulating endogenous stem cell pools or following exogenous transplantation. However, the efficacy of stem cell therapy is contingent upon the stem cells' ability to home and engraft in the injury site over an extended period. The purpose of this chapter is to discuss various strategies, such as preconditioning of stem cells (use of hypoxia, chemokine approaches, etc.),

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selecting the optimal time window, and route of delivery used to enhance stem cell homing ability in stroke.

Keywords

Stroke · Stem cells · Homing · Hypoxia · Chemokines

8.1 Introduction

Stroke is the third leading cause of death in low-income countries (Dai et al. 2020; Yan et al. 2007), and it is projected to remain the leading cause of disease burden worldwide (Zhou et al. 2019). The most common form of stroke (>85%) is ischemic stroke (Musuka et al. 2015). Regular blood flow to a particular brain region is hampered in this neurological pathophysiological condition, resulting in either no or inadequate glucose and oxygen supply levels. Hence, the affected area of the brain experiences neuronal cell death in a relatively short period, reducing the therapeutic window (Towfighi et al. 2011). tPA administration and thrombectomy are currently the only FDA-approved treatments for ischemic stroke, both of which have very narrow therapeutic time windows. The limited effectiveness of tPA administration and thrombectomy in stroke treatment necessitates developing alternative therapeutic methods to increase stroke survivors' disability-adjusted life years. Cell-based therapies have attracted the scientific community as a paramount technique for the intervention of stroke due to many advantages such as suitable isolation method, low immunogenicity that allows allogeneic transplantation without immunosuppressive drugs, trophic action, and ability to disintegrate into tissue-specific cell types (Sasaki et al. 2008; Zhang et al. 2007; Toma et al. 2002). Mesenchymal stem cells (MSCs), dental pulp stem cells (DPSCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), neural stem cells (NSC), very small embryonic-like stem cells, etc., have demonstrated therapeutic potential for stroke. Moreover, the patient-specific stem cells have revitalized the field of stroke research, thanks to the discovery of induced pluripotent stem cells (iPSCs). However, preclinical and clinical effectiveness with cell-based therapy has been limited. This may be due to insufficient homing, engraftment, cell survival, diminished regenerative capacity, and delayed administration.

8.2 Stem Cell Homing

In general, homing refers to a process in which cells move back to their original organ. In short, cell homing is a process in which the cells, residing at one place or floating in the peripheral blood, migrate toward the gradient of chemo-attractants released by a particular tissue and stay there for an extended period (Ratajczak et al.

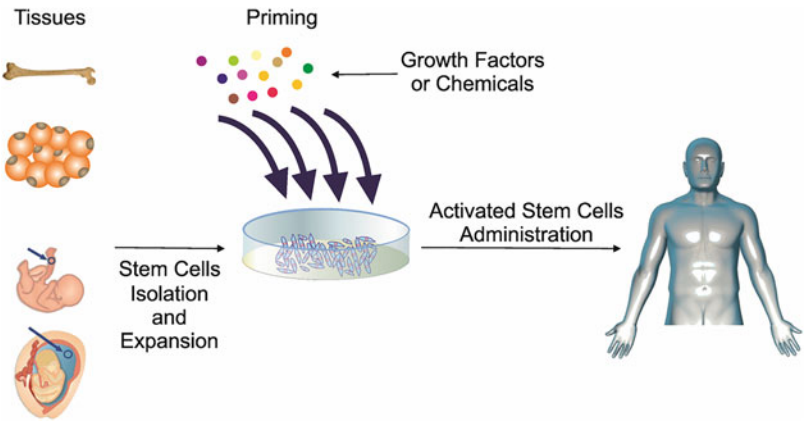

Fig. 8.1 A summary of the process of producing primed stem cells for the treatment of stroke. Steps involved in the production of primed stem cells: tissue source selection, stem cell isolation, stem cell expansion, stem cell priming, and stem cell application in stroke

2014; Bonig et al. 2007; Li et al. 2005). Despite decades of research, our understanding of molecular signals in homing remains a work in progress. We still don't have a complete understanding of the entire procedure (Hardy and Megason 1996). The majority of information about the processes underlying migration and homing comes from studies of leukocytes (Moser et al. 2001), HSC (Peled et al. 1999), and metastatic cancer cells (Muller et al. 2011) migration.

Stem cell homing is a term that refers to the ability of circulating stem cells or exogenous stem cells to locate and enter an environmental niche. Throughout its life, a stem cell may migrate between niches during embryonic development and adulthood. The stem cells sense the injury to the tissue and migrate to the area of injury to differentiate. The plasticity, differentiation, and migration of stem cells within a tissue are all dependent on the presence of specific signals within the damaged tissue's local microenvironment. As a result, the ischemic microenvironment is critical for stem cell migration, seeding, expansion, survival, renewal, growth, and differentiation during brain remodeling. While stem cell homing to the bone marrow has been extensively studied and established to be critical for normal fetal development, until recently, no ischemic-stem cell homing factor had been identified. To gain a better understanding of stem cell-mediated cell therapy for cerebral ischemia and possibly other ischemic-related diseases, it is critical to identify the signaling molecules that attract stem cells and direct their migration to damaged areas. Understanding the precise molecular mechanisms underlying stem cell mobility in response to local ischemic signals may provide new insights into how to manage stroke and other ischemic disorders more effectively (Fig. 8.1).

In stem cells, the homing process was firstly described for HSCs during clinical bone marrow transplantation (https://emedicine.medscape.com/article/208954-overview). HSCs and hematopoietic stem progenitor cells (HSPCs) are considered a well-established traveler, though the former being the best-studied cell in context to

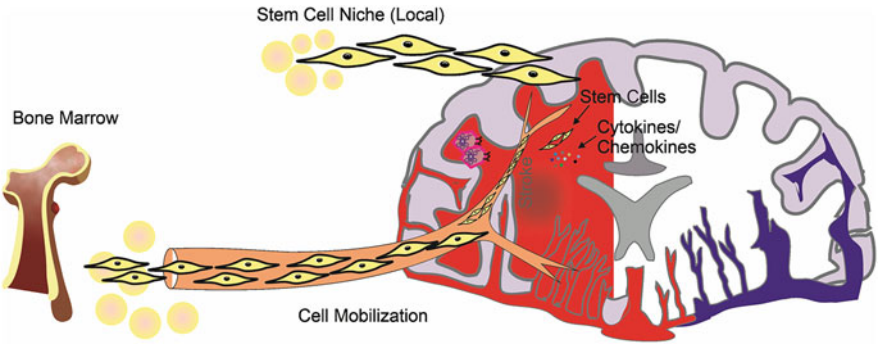

Fig. 8.2 A schematic illustration illustrating strategies for stem cells migration toward the areas of the infarct brain. In a nutshell, cells can migrate in response to concentration gradients. Chemokines and cytokines released by cells in the penumbra region can facilitate stem cell migration

homing. Apart from the two stated above, several other stem cell types, such as MSCs and neural stem cells (NSCs), etc., have shown migratory ability over time (Wang et al. 2020; Huang et al. 2018; Shiota et al. 2018; Tseng et al. 2018). HSC identifies "home" signals emanating from bone marrow stem cell niches, migrates to them, and repopulates the marrow (Tavassoli et al. 1990). HSCs travel from the aorta-gonad-mesonephros region through the fetal liver and spleen and then go to the bone marrow and spleen via the fetal liver (Durand et al. 2005). The pool of these is nourished and maintained, creating a "stem cell niche." As this niche includes the essential characteristics of what we call home, the HSPC migration to the bone marrow is usually considered homing (Hidalgo et al. 2008). Numerous surface ligands and receptors have been implicated in the process of homing. For example, HSPCs require selectin receptors, trans-membrane cell-surface proteins, with galactosyl and mannosyl specificities (Tavassoli et al. 1990). E-selectin (expressed by activated endothelial cells), P-selectin (on endothelial cells and platelets), and L-selectin (on leukocytes, including HPC) are the three selectins reported (Kansas et al. 1996).

In pathological states, the concept of "homing" is unambiguously different, with cells anticipated to move to the injured area, maintain, proliferate, differentiate, and replace the lost cells (Fig. 8.2). In stroke research, the physiological and regulatory mechanisms underlying the stem cell homing process are poorly understood (Karp et al. 2009). MSCs are the most widely studied cells in the field of stroke. Hence, there is a substantial amount of study in stroke on the processes by which MSC migrate to ischemic tissues and the role of cell surface receptors and chemicals in this process. In addition, endothelial cells' participation in MSC migration is highly studied in this field. According to consensus, injured cells produce particular chemokine ligands that direct transplanted cells' migration and infiltration into the ischemic area, similar to how monocytes are recruited to the site of inflammation (Sordi et al. 2009). Recent evidence suggests that ischemic injury induces the release of chemokines by astrocytes, microglia, and endothelial cells, hence stimulating

trans-endothelial migration of cells (Mao et al. 2017; Yan et al. 2007). The succeeding sections examine the issues connected with transplantation and the factors that enable transplanted cells in migrating and homing to the desired tissue:

8.3 Factors Influencing Stem Cell Homing

Timing and the route of delivery is a significant challenge for the success of stem cell therapy in transplantation biology that we will discuss in the next section. Apart from homing, the efficacy of stem cell therapy is highly dependent on the timing, route of delivery at the site or close to the site of injury, and engraftment into the injury site over a long period. The homing of the infused cells to the area of interest is a significant limitation, as, after administration of cells, the next crucial and challenging task is tracking cells from their site of administration to the target tissue (Wu et al. 2008; Lin et al. 2017). Further, various mechanical (shear stress, vascular cyclic stretching, and extracellular matrix stiffness are all hemodynamic forces that act on the vessel walls) and chemical (chemokines, cytokines, and growth factors, to name a few) factors have been determined which severely affect the homing process of infused cells to the damaged tissue (Wu et al. 2008). For example, the human body is the only source of cells for transplantation, and the yield is relatively low. After that, cells are expanded *in vitro* for a week or more to achieve a sufficient number of cells to achieve efficient homing and, as a result, an effective therapeutic dose (Hess et al. 2017). A lower number may lead to a diminished tropism toward the brain (Krause et al. 2019). During *in vitro* proliferation, the isolated cells have tended to lose their homing capacity to the targeted lesions (Gervois et al. 2016: Yavagal et al. 2014). In addition, the fact that most cells are stuck in the lungs and spleen after delivery is a significant drawback of this procedure (Gervois et al. 2016; Acosta et al. 2015). A strategy in this context would be to ensure that if the adhesion receptors on the surface of the cells are modified, they can be separated from the other organs such as the lungs and other peripheral organs. Schafer et al. (2020) modulated the cell surface structure of bone marrow-derived mesenchymal stem cells (BM-MSCs) using cationic molecules like polyethyleneimine (PEI) with or without nanoparticles. PEI treatment had no effect on MSC viability, immunomodulatory capacity, or differentiation potential; instead, it boosted CXCR4 expression and blocked their adhesion receptors in the lungs. When these cells were administered intravenously to stroke rats, the PEI-treated MSCs demonstrated significantly enhanced homing ability, reducing PEI-MSC adherent in the lung vasculature.

Notably, several modalities of stem cell infusion have been examined in a variety of animal models and a few clinical trials, including intracerebral (Chen et al. 2001), intracranial (Smith et al. 2012; Stroemer et al. 2009), intranasal (Wei et al. 2013), and stereotaxic infusion (Kameshwar et al. 2014). Surprisingly, all of these investigations found that the participants' functional outcomes improved. Therefore, a comparison of diverse routes has also been evaluated to determine a superior route of administration (Rodriguez-Frutos et al. 2016; Willing et al. 2013). However, there

was no evidence of considerable variance in clinical efficacy (Dulamea et al. 2015; Sun et al. 2015). As a result, the ideal method of cell infusion has yet to be discovered.

8.4 Timing of Administration of Cells

Cell treatment has the advantage of being able to change infusion schedule, which benefits a much larger patient population. According to the literature, effective stroke-to-transplantation periods vary greatly. Surprisingly, most preclinical investigations showed that transplanting took place during the first three days after a stroke (Locatelli et al. 2009; Guzman et al. 2008a). This time frame is already longer than the 3–6 h needed for t-PA treatment. Subacute (1 week after a stroke) and chronic (>3 weeks after a stroke) stem cell administration has been shown to improve recovery (Daadi et al. 2008; Shen et al. 2007). However, comparing the data to establish the best timing for transplantation is difficult because the research used different stroke models, cell types, cell delivery methods, and behavioral assessments to assess efficacy. This highlights the necessity of a more systematic and consistent approach to preclinical research, which allows for direct comparisons between investigations. The cell type may influence the best period for transplantation as well as the cell's mechanism of action. The timing of transplantation may also be affected by the delivery route. Intravascular transplantation may need early administration because stem cells employ inflammatory signals to travel to the wounded brain (Guzman et al. 2008a, b), yet stem cells were also found in the brain after late intravascular delivery (1 month post-stroke) (Shen et al. 2007). Intraparenchymal cell injection, on the other hand, would benefit from being injected later, after the first inflammatory response has subsided, because this would boost cell survival (Kelly et al. 2004). We may be able to employ non-invasive imaging tools to determine the best timing for transplantation on a patient-by-patient basis once we have a better knowledge of how cells interact with the brain and vice versa.

8.5 Optimizing the Route of Administration of Cells

The efficacy, bioavailability, and utility of a pharmacological medication depend on the administration method. Thus, to maximize the stability and therapeutic efficacy of transplanted stem cells, the mode of administration should be optimized to facilitate the cells' homing to the target tissue. It is difficult to determine the optimal route of cell transplantation in ischemic stroke therapy because multiple trials have used various stroke models, cell types, cell infusion methods, and efficacy assessment methods. At the moment, cell-based experimental and clinical research for stroke therapy employs two distinct delivery methods: local delivery and systemic infusion. If the primary goal of treatment is neuroprotection, acute administration is preferred. However, if the primary goal is tissue repair, subacute delivery is recommended (Carmichael 2006). Local delivery has the advantage of directly

targeting the cells in damaged areas of the brain; however, this method requires general anesthesia and neurosurgical procedures, limiting clinical applications and may result in additional brain damage due to the high degree of invasiveness (Carmichael 2006). Subacute delivery would be advantageous if the cells enhance endogenous repair mechanisms (e.g., plasticity and angiogenesis), as these events occur more frequently in the first few weeks after ischemia (Carmichael 2006).

In systematic infusion, cells remain close to oxygen- and nutrient-rich blood vessels and are particularly useful when injecting directly into damaged areas is difficult or impossible. Systemic delivery using intravenous (IV) or intra-arterial (IA) injections is a less invasive approach. The IV delivery method enhances cell migration and homing in the damaged brain (Walczak et al. 2008; Nomura et al. 2005; Akiyama et al. 2002; Horwitz et al. 1999; Pereira et al. 1998). However, a significant disadvantage of IV stem cell delivery is significant cell entrapment in the capillary beds of other organs such as the lungs, liver, spleen, and kidneys leaving only a small number of cells reaching the brain (Ankrum et al. 2010; Fischer et al. 2009; Hauger et al. 2006; Kraitchman et al. 2005; Barbash et al. 2003). The disadvantage of IA infusion is that it may result in "microvascular occlusions," resulting in decreased blood flow to the brain and micro-embolization of the cerebrovasculature (Savitz et al. 2011; Walczak et al. 2008; Barbash et al. 2003). Additionally, some cells from the target organ that approach the capillaries or microvessels become passively trapped (Karp et al. 2009; Sackstein et al. 2008; Walczak et al. 2008). The other significant disadvantage of this method is that cells require time (up to ten days) to transmigrate from the endothelium into the tissue parenchyma following entrapment in the vasculature (Karp et al. 2009; Sackstein et al. 2008; Walczak et al. 2008).

In recent years, there has been a focus on non-surgical options capable of successfully delivering cells into the ischemic brain area, thereby increasing the capacity and protection of cell transplantation (Wagner et al. 2009). One such option is cell administration through the nasal cavity, which is a highly effective method of drug delivery that bypasses the BBB and delivers therapeutics directly to the CNS (Dhuria et al. 2010; Thorne et al. 2004). It has been observed that when cells are infused through the intranasal route, the brain accumulates the most significant number of cells compared to the other investigated organs, including the heart, kidney, lung, liver, spleen, and stomach (Danielyan et al. 2011). Intranasal administration of drugs has been used for decades; it is successfully tested in a Parkinson's disease model (Danielyan et al. 2011), a neonatal hypoxic-ischemic stroke model (van Velthoven et al. 2010), and in normal rats and mice (Danielyan et al. 2011). Wei et al. (2013) demonstrated that intranasal transplantation could be successfully performed up to 24 h after ischemia while maintaining neuroprotective effects in the post-ischemic brain. Intranasal administration of BM-MSCs in healthy rats demonstrates that the cells can migrate into various brain zones via olfactory and trigeminal nerve pathways and enter the cerebrospinal fluid (CSF) via the cortex surface, and then enter the brain parenchyma (Danielyan et al. 2011).

8.6 Preconditioning of Cells for Improved Homing

Preconditioning has sparked a lot of curiosity in transplantation biology as stem cells can be considerably enhanced before infusion through preconditioning approaches, resulting in better *in vivo* outcomes. Incubation with pharmacological/chemical agents or trophic factors/cytokines, hypoxia preconditioning, and gene editing, etc., are some of the prime strategies to improve stem cells' proliferative, secretory, migratory, and differentiation capacities *in vitro* and *in vivo*.

8.7 Hypoxia Preconditioning

Following infusion, the fate of stem cells is strongly influenced by the microenvironment. When transplanted stem cells are exposed to a harsh microenvironment resulting from the pathological state, their regenerative capacity and secretome are compromised. Hypoxia, heat, nutritional deprivation, oxidative stress, and inflammation are all common cues in a harsh microenvironment (Zhao et al. 2020; Abdelwahid et al. 2016). Studies concluded that stem cell-based therapy's limited clinical efficacy might be due to poor homing, engraftment, poor cell survival, impaired regenerative ability, and delayed administration. It is proposed that pre-treatment of the cell with hypoxia before transplantation into the stroked brain is beneficial (Fig. 8.3). Observations indicate that when cells are cultured in hypoxic conditions (typically 1–4% O_2), several functions, including growth factor receptors' expression and migratory ability, get significantly unregulated (Rosova et al. 2008). For example, within 1.5 h of administration of hypoxia-preconditioned BM-MSCs intra-nasally to male C57BL/6 mice with induced focal cortex ischemic stroke, the cells reached the ischemic cortex and accumulated outside vasculatures. An increased expression of migration-related proteins such as CXCR4, matrix metalloproteinase 2 (MMP-2), and matrix metalloproteinase 9 (MMP-9) was observed in hypoxia-preconditioned BM-MSCs. Intriguingly, the renal ischemia-reperfusion injury results showed that the administration of 1% O_2 MSCs significantly ameliorated renal fibrosis and inflammation in rats with ischemia-reperfusion injury, compared to the administration of 21% O_2 MSCs. Additionally, it is observed that 1% O_2 MSCs could upregulate humoral factors such as VEGF, HGF, and PGE2. VEGF is a critical factor in HGF production and the anti-fibrotic effect of 1% O_2 MSCs.

Compared to non-preconditioned BM-MSCs, the cells demonstrated enhanced migratory capability *in vitro* and dramatically increased homing efficiency *in vivo* (Wei et al. 2013). Similarly, Wang et al. (2017) transplanted hypoxia-primed BM-MSCs into a rat model of global cerebral ischemia induced by cardiac arrest and observed that the hypoxia-primed BM-MSCs migrate and integrate into the ischemic cortex, thereby reducing neuronal death and inflammation. Additionally, they demonstrated that primed MSCs migrate and integrate via the PI3K/AKT and HIF1/CXCR4 pathways. HIF-1α and HIF-1β are critical in preconditioning for ischemia and neuroprotection against ischemia. The translocation and activation of

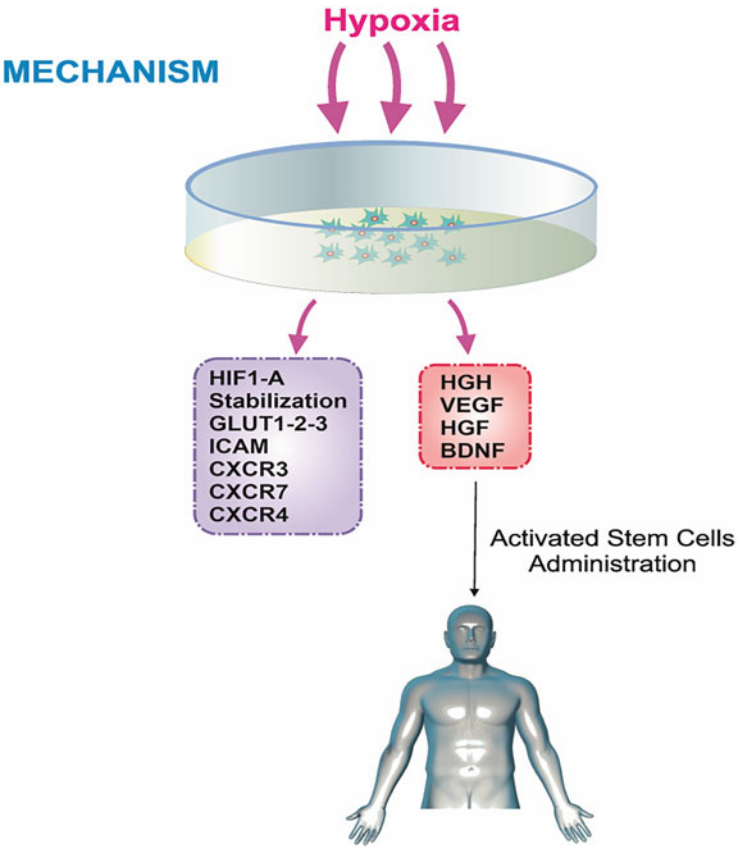

Fig. 8.3 Hypoxia priming strategies to enhance stem cell therapeutic efficacy are depicted schematically. In continuous-line boxes, post-priming released soluble factors are depicted. The current diagram depicts the activation of cells in response to hypoxia and their subsequent transplantation into stroke patients

HIF-1 in the nucleus result in enhanced expression of several downstream genes, such as VEGF, EPO, NCX-1, PDK-1, LDHA, and uncoupling protein-2 (UCP-2). These proteins signals for survival, balances ion homeostasis, regulates oxidative stress and glycolysis in mitochondria, and regulates a wide variety of stress-induced responses, thus possibly enhancing stem cell survival during ischemic conditions. Thus, hypoxia-preconditioned MSCs are useful for allogeneic cell therapy in other therapies of ischemic-reperfusion diseases.

8.8 Growth Factors

In models of ischemic brain injuries, the ischemic microenvironment may express signals that promote the recruitment of circulating stem cells to the ischemic area, where they can differentiate into mature brain tissue. In addition, it has been noticed that trophic factors are released due to ischemic damage to brain tissue, directing stem cells toward the damaged tissue. In ischemic rat brains, a variety of neurotrophic factors are released, resulting in the production of human BM-MSC growth factor (Huang et al. 2017; Azad et al. 2016). Granulocyte colony-stimulating factor (G-CSF) is the dominant growth factor regulating granulopoiesis and promoting survival, proliferation, functional activation, and maturation of neutrophil lineage cells, besides capable of mobilizing bone marrow stem cells into the peripheral blood. Recent studies show that subcutaneous G-CSF injections lead to BM-MSC migration to the injured brain and assist in neural repair by helping to mobilize BM-MSCs (England et al. 2012; Sprigg et al. 2006).

Further, it has been documented that following MCAO, GDNF infusion increased cell proliferation in the ipsilateral SVZ, recruited new neuroblasts to the striatum, and improved the survival of newly mature neurons (Kobayashi et al. 2006). Numerous studies have demonstrated that growth factors play a critical role in stroke recovery, in addition to their involvement in the maintenance of a variety of stem cells. Intraventricular medium B contains the growth factors EGF and fibronectin and a proliferation and adhesion promoter. Chen et al. (2020) discovered that when medium B was infused into the ventricles immediately following a stroke, neural progenitor stem cells proliferation, migration, and neuronal differentiation increased while inhibiting cell apoptosis.

Integrins' effect on stem cell homeostasis and reparative efficiency has also been studied in several studies. Integrins are a family of cell surface ligands required for cell migration and are expressed on adipose-derived MSC-like cells (De Ugarte et al. 2003). VLA4 is a cell adhesion molecule found on the plasma membranes of leukocytes. VLA4 and VCAM1 (vascular cell adhesion molecule-1) interactions are required for stem cell migration (Nitzsche et al. 2017; Brunner et al. 2013). Integrin-neutralizing antibodies show that integrin-beta1 inhibits MSC homing to infarcted myocardium but not integrin-alpha4 (Ip et al. 2007). Other studies, however, have found that integrin-alpha4 plays a role in MSC migration (Ruster et al. 2006).

8.9 Chemical and Pharmacological Priming

Pre-treatment of stem cells with approved chemicals or pharmaceuticals is a time-saving and straightforward strategy to improve cell homing. Numerous studies demonstrate that priming MSCs with chemicals or pharmacological compounds enhances their proliferation, migration, and differentiation capabilities *in vitro*, as well as their therapeutic potential in a variety of disease states when applied *in vivo* (Zhou et al. 2015; Müller et al. 2011; Tsai et al. 2011). Valproate, a sodium salt of

valproic acid, a drug commonly used to treat bipolar disorder (Tsai et al. 2010), has been shown to induce hMSCs migration. When it was used to prime MSCs before transplantation in an MCAO rat model, it increased the cells' ability to homing. On the other hand, lithium precondition to MSCs has similar consequences. When these substances were combined and used to prime MSCs, the cells' homing ability improved significantly before transplantation in an MCAO rat model. Further findings revealed an enhancement in CXCR4 and MMP9 expression (Tsai et al. 2011). It has been demonstrated that peptides can be successfully targeted (Qin et al. 2012; Li et al. 2011). Huang et al. (2017) used this approach to investigate the effect of peptide-modified MSCs overexpressing miR-133b on homing and therapeutic efficacy in the MCAO rat model. MSCs were covalently coated with peptides modified with palmitic acid (lipid) and CLEVSRK (FITC) NC (peptide). Following transplantation, co-modified cells (cell surface modification with a non-viral miR-133b overexpression system) demonstrated enhanced homing and therapeutic efficacy. Interestingly, MSCs preconditioned with tetramethylpyrazine – a chemical derived from the medicinal herb *Ligusticum wallichii* (a.k.a. Chuan Xiong) – demonstrated migratory potential in ischemic rodents. In a recent report, Li et al. (2017) preconditioned the BM-MSCs with tetramethylpyrazine. They found that preconditioning increased the CXCR4 expression, improved the migration and homing ability of BM-MSCs, thus facilitating blood vessel growth and offering therapeutic benefits for neurological conditions. Ethionamide preconditioning increased CXCR4 and CXCL12 expression in MSCs while promoting MSCs proliferation (Lee et al. 2020). Moreover, H_2O_2-induced preconditioning increased the migration of MSCs through activation of CXCR4 and ERK-induced expression of cMet, which binds to HGF, allowing MSCs to migrate and participate in healing.

Though most preclinical studies used MSCs for transplantation, administering NSCs may have a similar effect on stroke recovery. Sakata et al. (2012) used minocycline to reprogram NSCs before transplanting them into an MCAO rat model. The primed NSCs demonstrated an increased capacity for survival in an *in vitro* stroke model (oxygen-glucose deprivation), as well as increased paracrine abilities, including the release of BDNF, NGF, glial cell-derived neurotrophic factor, and VEGF. When these activated cells were administered to a stroke model *in vivo*, they migrated extensively to the damaged area, significantly reducing infarct volume and improving neurological performance compared to unconditioned NSCs.

8.10 Chemokines: SDF1/CXCR4

Chemokines are a class of small proteins that can attract immune cells through chemotactic mechanisms (Cheng et al. 2017). Around 50 chemokines and 18 receptors comprise the human chemokine system (Moser et al. 2001; Loetscher et al. 2000; Murphy et al. 2000). Chemokines are classified into four structural families based on the number and spacing of cysteine residues in the NH2-terminal region (CXC, CC, CX3C, and C). Inducible and constitutive (also called homing, lymphoid, or housekeeping) chemokines have recently been reclassified. The

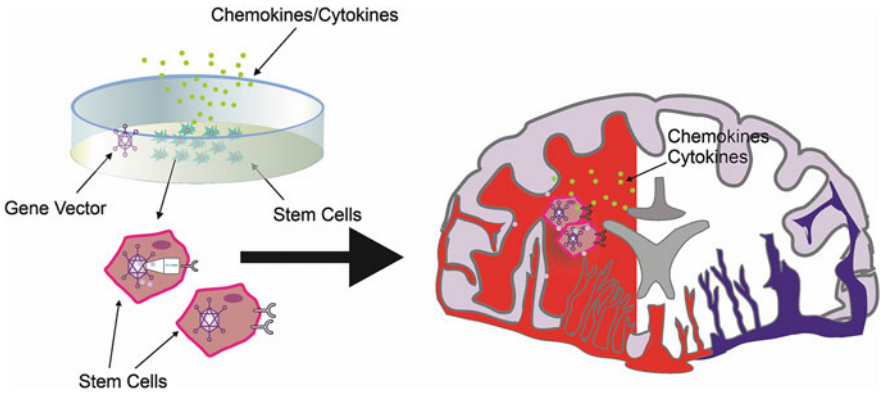

Fig. 8.4 Preconditioning stem cells in culture with growth factors, either transiently or permanently, via lentiviral knock-down, and delivering the primed stem cells to the stroke brain

following are just a few examples of the chemokines: RANTES (regulated upon activation), epithelial-neutrophil activating protein-78 (ENA-78 or CXCL5), monocyte-chemoattractant protein-1 (MCP-1 or CCL2), and macrophage inflammatory protein-1α (MIP-1α or CCL3). One candidate chemotactic molecule is stromal cell-derived factor (SDF)-1α, also referred to as CXCL12, which is extensively studied in homing studies. It is a member of the alpha CXC chemokine subfamily and was initially defined as a pre-B cell growth-stimulating factor (Sierra et al. 2004). SDF1 is constitutively expressed in neurons, glial cells, endothelial cells, and meningeal cells in a mature CNS, while one of its receptors, CXCR4, is constitutively expressed in neurons, astrocytes, microglia, and ependymal cells (Banisadr et al. 2002; Stumm et al. 2002). SDF-1 and its receptors are involved in various physiological and pathological processes, including inflammatory processes, neurogenesis, and angiogenesis in the central nervous system (CNS) (Cheng et al. 2017) (Fig. 8.4).

Due to the high expression of chemokine receptors, matrix metalloproteinase (MMPs), and adhesion molecules, stem cells can migrate to injury sites (Galipeau et al. 2016). Previous research has shown that after a stroke, HSC/HPCs migrate from the bone marrow to the peripheral circulation (Deb et al. 2010; Shyu et al. 2008) and that they migrate selectively into ischemic areas to aid in the recovery of tissue plasticity and function (Orlic et al. 2001). There is evidence that increased circulating HSC/HPCs improved neurological function following stroke, indicating that HSC/HPCs may play a critical role in mitigating stroke damage and promoting healing following stroke (Hochsmann et al. 2009; Chu et al. 2008;Rouhl et al. 2008; Sobrino et al. 2007). Hill and colleagues demonstrated that SDF1 expression increases in ischemic regions, particularly in the penumbra, following focal ischemic stroke and remains elevated for up to 6 weeks (Hill et al. 2004). As with SDF1, CXCR7 was found to significantly increase in the peri-infarct region (Schonemeier et al. 2008). SDF-1 overexpression recruits $CXCR4^{+}$ stem/progenitor cells to ischemic tissue, including hematopoietic stem cells (HSPCs) (Lanfranconi et al. 2011;

Dar et al. 2006), bone marrow mesenchymal stem cells (BM-MSCs) (Wang et al. 2008; Hill et al. 2004), and endothelial progenitor cells (EPCs). Recently, two studies involving human stroke patients reported an increase in the serum level of SDF-1 following stroke. The change in serum SDF-1 was found to be positively correlated with infarct volume and severity of stroke (Duan et al. 2015; Liu et al. 2015). When the SDF1/CXCR4/CXCR7 axis was used to engineer the MSCs due to its ability to attract cells roaming in the peripheral blood to the injury site, Yu et al. (2012) demonstrated increased mobilization and neuroprotection of CXCR4-overexpressing rat-MSCs in the MCAO rat model. Wang et al. (2014) used CXCR7-overexpressing MSCs in a transient cerebral ischemia-reperfusion model in a similar study. It was demonstrated that both SDF1 receptors, CXCR4 and CXCR7, were expressed in rat-BMMSCs and promoted BM-MSC migration synergistically. Besides, Andres et al. showed that CCL2/CCR2 interaction is needed for the therapeutic homing of NSCs injected intravenously. Interestingly, these interaction molecules are lost during the isolation and processing of separated cells for transplantation, resulting in impaired homing capacity (Andres et al. 2011). Taken together, these findings suggest that the SDF-1/CXCR4/CXCR7 signaling axis contributes significantly to the amelioration of ischemic stroke outcomes, most likely via neurogenesis and angiogenesis.

8.11 Stem Cell Tracking

Monitoring the migration of transplanted stem cells *in vivo* is frequently accomplished by labeling cells with a contrast agent such as superparamagnetic iron oxide nanoparticles (SPION), quantum dots, and so on, and then scanning them *in vivo* with molecular imaging. *In vivo* imaging methods for cell tracking in clinical and preclinical studies is critical for the development and optimization of stem cell treatment. For a successful outcome, the cell type, time, dosage, and method of administration, and the safety and biocompatibility of the tracking agents must all be considered. Thanks to inventions and daily advances in *in vivo* imaging technologies, real-time monitoring of stem cell survival, proliferation, and differentiation are now possible.

Molecular imaging techniques used to follow transplanted cell migration in vivo include magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), near-infrared fluorescence (NIRF) imaging, and bioluminescence imaging (BLI) (Nucci et al. 2020; Rizzo et al. 2017). Each one has its own set of benefits and drawbacks. MRI includes the fact that it does not use radiation and has no tissue penetration limit. Furthermore, MRI has a time resolution of minutes to hours and a spatial resolution of 0.02–0.1 mm. However, it has low sensitivity, low contrast, a high cost, and a long scanning time. It has been observed that over time the intensity of MRI to detect transplanted cells decreases. For example, when SPIO-BMSCs were injected in MCAO rats, a gradual reduction in intensity over time was recorded (Huang et al. 2019). The SPIO-BMSC (traceable for 30 days) revealed hypointense lesions. The same signal

intensity was observed in bone marrow mesenchymal stromal cells (BMSCs) transduced with a lentivirus containing a shuttle plasmid (pCDH-CMV-MCS-EF1-copGFP) containing the ferritin heavy chain 1 (Fth1) gene.

PET is another viable option for investigating stem cell homing in stroke research, with the advantages, including increased sensitivity, excellent penetration depth, and an entire body image. The spatial resolution of PET is 1–2 millimeters, and the temporal resolution is seconds to minutes. Daadi et al. (2013) used genetic engineering to create NSCs that express monomeric red fluorescence protein and herpes simplex virus truncated thymidine kinase for multimodal molecular imaging. The cells were also labeled with SPION for MRI examination. The MRI and PET were used to track the fate and function of grafted cells in real-time for three months. PET imaging revealed that grafted animals showed increased metabolic activity and that transplanted cells were functioning in vivo. The immune-histopathological findings demonstrated that grafted NSCs were distributed in the stroke-lesion parenchyma and differentiated into neurons, astrocytes, and oligodendrocytes during a 3-month survival period. The high cost of the required cyclotron, as well as radiation exposure, is its drawbacks.

SPECT has a spatial resolution comparable to PET. Its advantages include high sensitivity and the absence of a tissue penetration limit or the need for a cyclotron, while disadvantages include radiation exposure and difficulties quantifying the results. For the treatment of stroke patients, SPECT is a valuable tool. Preliminary data show that SPECT could potentially predict an early stroke after a transient ischemic attack and distinguish a lacunar stroke from a cortical stroke (Masdeu and Brass 1995). Early SPECT after an acute stroke is more accurate than computed tomography or magnetic resonance imaging in depicting the area of ischemia. SPECT can be used to more easily document the reperfusion of an arterial territory following thrombolysis than angiography.

In preclinical stroke research, NIRF imaging and BLI are widely used modalities (Xu et al. 2020; Jang et al. 2010). The advantages associated with these two are modest spatial resolutions, ranging from 2–3 mm to 3–5 mm, respectively. The temporal resolution of both systems ranges from seconds to minutes. The benefits of NIRF imaging and BLI include high sensitivity, no exposure to radiation, low cost, and the ability to be activated. Furthermore, BLI has the advantage of simple equipment operation and non-damaging imaging. In contrast, both optical imaging techniques have the disadvantages of sensitivity attenuation by overlying tissues and insufficient penetration depth. To summarize, molecular imaging modalities such as nuclear imaging (PET and SPECT) and magnetic resonance imaging (MRI) have a wide range of potential uses in *in vitro* and preclinical research, and are promising tools in stem cell therapy.

8.12 Conclusion

Clinical studies report that stem cell therapy helps to treat patients with ischemic stroke. Issues such as targeted targeting of the ischemic brain, effectiveness in vivo, and benefits without adverse effects remain a focus of studies. Since stem cells have a unique affinity and capacity to move into the brain, they may be useful both therapeutically and for facilitating drug delivery. It will be necessary to develop methods that combine optimized delivery, dosage, preconditioning, and tracking information for investigating stem cells fate in ischemic stroke.

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Role of MicroRNAs in Stroke Pathology and Recovery

9

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Abstract

Ischemic stroke is a leading cause of morbidity and mortality all over the world. Stem cell-based therapies have become a popular choice with scientists and healthcare providers because they could help stroke patients. The fact that several clinical studies have shown that a lack of effective neurorestorative approaches is a barrier to stem cell therapy success and should be addressed. MicroRNA (miRNA) are small non-coding RNA molecules that suppress gene expression, and have emerged as novel therapeutic targets in stroke. In this chapter, we discuss the role of microRNAs in stroke recovery, as well as how they are regulated and how they affect recovery. The use of microRNAs (miRNAs) as a therapeutic agent to protect against stroke pathology is also presented and discussed in this article.

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Ischemic stroke · Stem cells · MicroRNA · Blood-brain barrier · Small non-coding RNA · Neurogenesis

9.1 Introduction

Stroke is the leading cause of death and serious, long-term disability, with significant economic consequences. When blood flow is interrupted, a complex cascade of pathophysiological events occurs at the blood-vascular-parenchymal interface that evolves over time, resulting in neural cell damage and edema formation. Cerebral ischemic injury causes a significant and detrimental increase in inflammation, as well as multiple cell death pathways, but it also causes a series of events associated with regenerative responses, such as vascular remodeling, angiogenesis, and neurogenesis. The primary goal of stroke therapies today is to assist in the rapid recanalization of obstructed blood vessels, though the current treatment effect of stroke is not ideal (Malhotra et al. 2017). As a result, novel stroke treatment strategies are immediately required. Emerging evidence suggests that epigenetic reprograming may be an important event in the ongoing post-stroke changes and recovery.

Non-protein-coding regions make up at least 98% of the human genome. The vast majority of these regions are transcribed into RNAs. Transcripts from these regions were previously thought to be transcriptional noise or background activity; however, because of their expression, function, and mechanism of action, these non-coding RNAs have recently received a lot of attention. Non-coding RNAs (ncRNAs) are a type of genetic, epigenetic, and translational regulator that includes microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). miRNAs, which are short molecules with lengths ranging from 21 to 25 nucleotides, are a common and evolutionary stable class of endogenous ncRNAs. They inhibit translation and degrade the corresponding mRNA through imperfect or near-perfect base pairing, primarily to the target mRNA's untranslated region of 30 bases (UTR). lncRNAs, which are typically defined as having a length of more than 200 nucleotides, are cell and tissue-specific. They are classified based on their function, genomic location between gene coding regions (long intergenic ncRNAs), or ability to overlap genes in either sense or antisense directions (Esteller 2011). In the nucleus, long non-coding RNAs (lncRNAs) act as guides for chromatin-modifying complexes and transcription factors. Circular RNAs (cirRNAs) have been extensively studied in cells for their signaling and regulatory roles. Briefly stated, it is known that all of these regulate transcription and translation processes. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke, and amyotrophic lateral sclerosis have been found to contain dysregulated miRNAs, implying that disruptions in the miRNA regulatory pathway may play a role in the development of these diseases. Because of this, it remains unclear what molecular mechanisms underlie the pathological consequences

of dysregulated microRNAs. Multiple stages of neurogenesis have been demonstrated to be regulated by microRNAs (miRNAs), extending from the proliferation of neural stem cells through the differentiation and maturation of neurons. A growing body of research indicates that ncRNAs (particularly miRNAs and lncRNAs) are involved in the pathogenic processes associated with cerebral ischemia and post-stroke recovery (Wang et al. 2018; Bai et al. 2018; Zhang et al. 2017; Mehta et al. 2017; Dharap et al. 2009, 2012). This chapter discusses the role and the functions of miRNAs and their role in stroke brain rehabilitation.

9.2 MiRNA Biogenesis and Function

MiRNA biogenesis begins with the post- or co-transcriptional processing of RNA polymerase II/III transcripts (Ha and Kim 2014). MiRNAs are sometimes transcribed as a single long transcript called a cluster, which may have similar seed regions and is referred to as a family (Kim and Kim 2007). MiRNAs are processed primarily from introns and a few exons of protein-coding genes, with approximately half of all currently identified miRNAs being intragenic. The remaining genes are intergenic, meaning that they are transcribed independently of the host gene and under the control of their own promoters (de Rie et al. 2017; Huang 2017). MiRNA biogenesis can be divided into two categories: canonical pathways and non-canonical pathways.

9.3 Canonical Pathway

MiRNA genes are transcribed as large polyadenylated RNA molecules known as primary miRNAs (pri-miRNAs) by RNA polymerase II in this pathway (Davis and Hata 2010). In short, the complementary sequence regions of these pri-miRNAs are capable of forming stem loops with bulges and mismatches. Prim-miRNAs are processed in the nucleus by a protein complex containing the RNase III enzyme double-stranded RNA-specific endoribonuclease nuclear type III (DROSHA, also known as RNASEN) and the DGCR8 microprocessor complex subunit, which is part of the DiGeorge syndrome critical region 8 (DGCR8) microprocessor complex (Gregory et al. 2006; Denli et al. 2004). Pri-miRNAs are degraded into smaller double-stranded RNA (dsRNA) molecules, which are referred to as pre-microRNAs, as a result of this degradation process. In mammals and other vertebrates, exportin 5 (XPO5) transports pre-miRNAs to the cytoplasm (Lund et al. 2006; Ohrt et al. 2006). Pre-miRNAs are cleaved and stripped of their loops in the cytoplasm by the RNase III enzyme DICER, which, through interaction with the protein TRBP, results in the production of dsRNAs with overhangs of 2–3 nt at both ends (Daniels et al. 2009; Chendrimada et al. 2005). Because mature functional miRNAs are single-stranded, these processed products are referred to as miRNA duplexes. The RNA-induced silencing complex (RISC), a ribonucleoprotein complex capable of unwinding both strands, must actively participate in the final processing step.

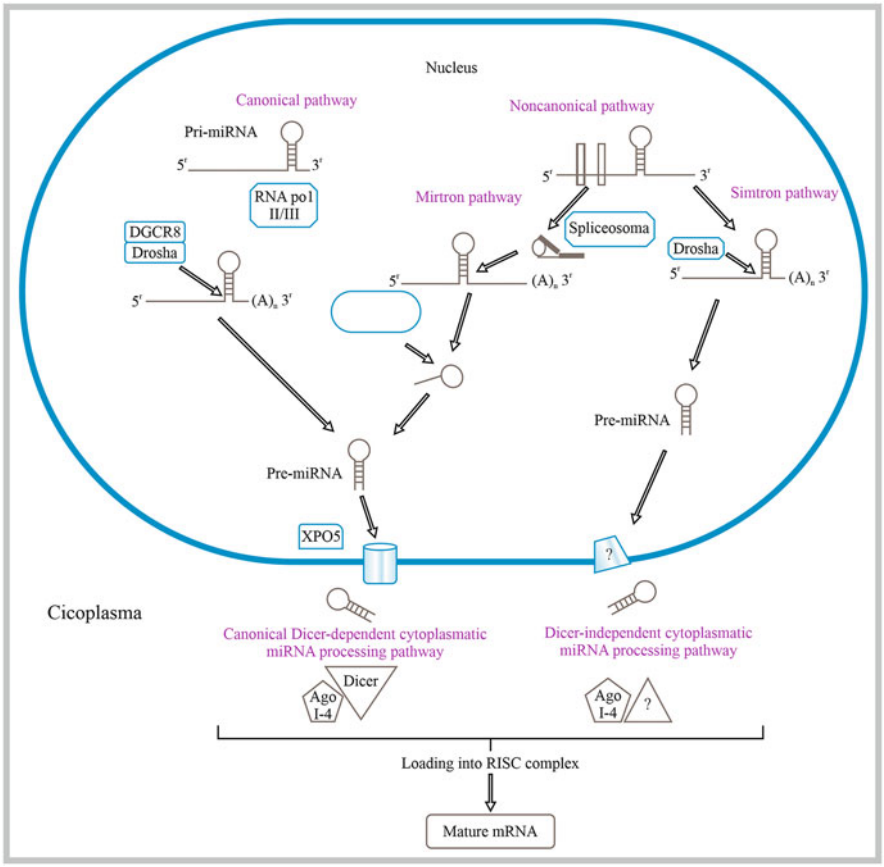

Fig. 9.1 Illustration of canonical and non-canonical miRNAs biogenesis

However, even though either strand of the miRNA duplex is capable of functioning, only a single mature miRNA is incorporated into the RISC complex to induce mRNA silencing (Okamura et al. 2009; Shin 2008). RISC mediates the recognition of the targeted mRNA once it has been loaded (Fig. 9.1).

9.4 Non-canonical Pathway

Non-canonical miRNA biogenesis pathways make use of various combinations of the proteins that are part of the canonical pathway, such as Drosha, Dicer, exportin 5, and so on. Non-canonical miRNA biogenesis can be divided into two categories: miRNA biogenesis that is Drosha/DGCR8-independent and miRNA biogenesis that is Dicer-independent. The Drosha/DGCR8-independent pathway generates pre-miRNAs that resemble Dicer substrates. The 7-methylguanosine (m7G)-capped

pre-miRNA is an example of such pre-miRNAs (Babiarz et al. 2008; Ruby et al. 2007). Exportin 1 transports these nascent RNAs directly to the cytoplasm, bypassing the requirement for Drosha cleavage. The m7G cap, which inhibits 5p strand loading into Argonaute, is most quick to attribute for the significant 3p strand bias (Xie et al. 2013). Drosha, on the other hand, processes Dicer-independent miRNAs from endogenous short hairpin RNA (shRNA) transcripts. Because they are too short to be Dicer-substrates, these pre-miRNAs require AGO2 to complete their maturation within the cytoplasm (Yang et al. 2010). This, in turn, promotes AGO2-dependent slicing of the 3p strand and loading of the entire pre-miRNA into AGO2. The trimming of the 5p strand at 3′–5′ completes their maturation (Cheloufi et al. 2010).

9.5 Neurogenesis in Stroke

Neurogenesis was thought to be impossible before the discovery of neural stem cells (NSCs) in the brain. Sally Temple's research in the subventricular zone of the mouse brain in 1989 identified multipotent, self-renewing progenitor and stem cells (Shen et al. 2004). Since then, studies have shown the presence of NSCs in many parts of the brain, including the subgranular zone (SGZ) of the hippocampus, as well as in the subventricular zone (SVZ) (Koh and Park 2017). In this context, stroke has been linked to a neurogenic burst (Kernie and Parent 2010; Arvidsson et al. 2001). These intriguing findings suggested that the neurogenic burst is an adaptive response that promotes brain recovery through the replacement of lost neurons (Cuartero et al. 2021). The SVZ neuroblasts move into the injured striatal region and take on projection neuron properties (Arvidsson et al. 2001; Parent et al. 2002; Jin et al. 2003). Interestingly, stroke causes deficits in hippocampus-associated spatial memory (Yonemori et al. 1999), and the increase in SGZ neurogenesis may be intended to compensate for these cognitive impairments. Striatal neurogenesis may thus aid in the recovery of stroke-impaired motor function. With age, the number and capacity of NSCs decrease. As a result, it is critical not only to protect NSCs from various insults, but also to have a diagnostic estimate in addition to the reparative mechanisms. Several recent studies have suggested that detecting dysregulated microRNAs could be used to diagnose and treat nervous system disorders. Hence, miRNAs could be an answer that could be used as a diagnostic marker besides enhancing the reparative properties of these cells. In summary, exploring whether the ischemic brain retains the capability for stroke-induced neurogenesis and the factors involved in enhanced neurogenesis is therefore of great clinical importance (Fig. 9.2).

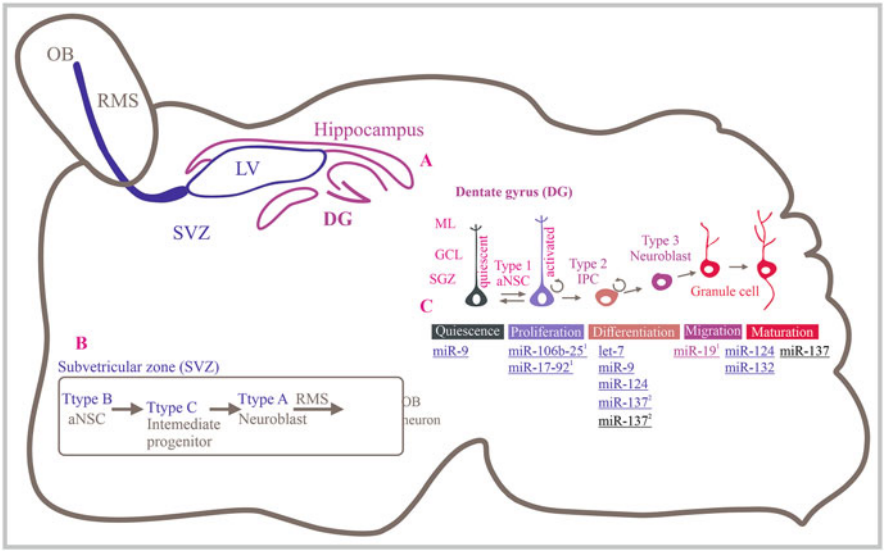

Fig. 9.2 MicroRNAs (miRNAs) are important regulators of the multistep process of adult neurogenesis. The above diagram depicts the role of miRNAs in the regulation of NPSCs

9.6 MiRNAs and Stroke

In previous research, circulating microRNAs were proposed as diagnostic and/or prognostic markers in a variety of cardiovascular diseases (Eyileten et al. 2018; De Rosa et al. 2018). However, only a few research studies on the role of miRNAs in central nervous system (CNS) disorders have been demonstrated (Bushati and Cohen 2008; Nelson et al. 2008). Practically, miRNAs play crucial roles in each phase of brain function, comprising neurogenesis (Li and Jin 2010), neural development (Cochella and Hobert 2012), and cellular responses controlling modifications in synaptic plasticity. Circulating microRNAs have the potential to be used as clinical biomarkers, similar to a liquid biopsy collected from peripheral blood that provides information on the pathophysiological processes taking place in the stroke brain (Di Ieva et al. 2014; De Rosa and Indolfi 2015; De Rosa et al. 2014). An advantage of miRNA-based therapies is that endogenous miRNA levels can be altered in the clinic using synthetic mimics and inhibitors (Janssen et al. 2013; Bader 2012). The level of miR-210 can be used to predict the outcomes of stroke (Tan et al. 2009). Data from the next-generation sequencing studies in rats in the first miRNA expression profiling study in cerebral ischemia, which used microarray analysis and real-time quantitative PCR techniques, revealed that a total of 236 microRNAs (miRNAs) were identified in brain and blood samples collected

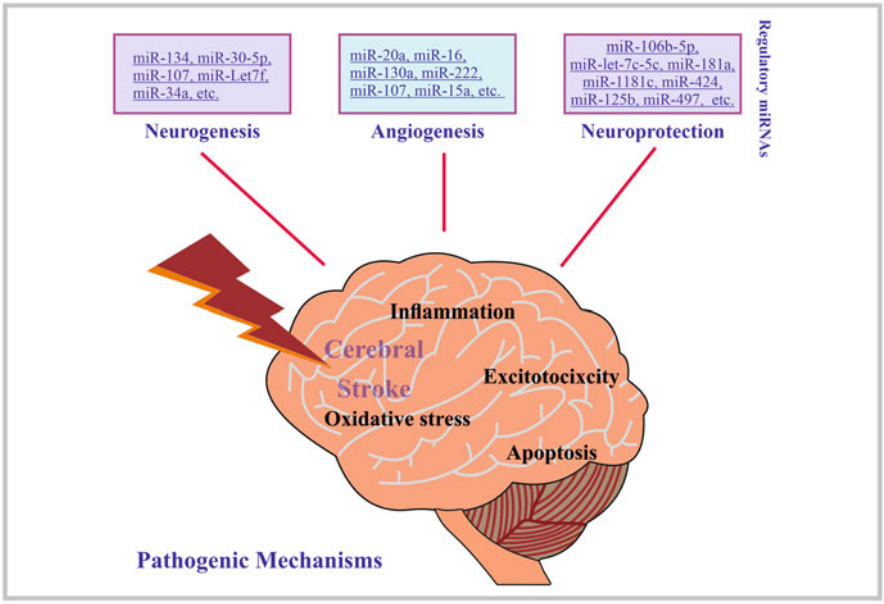

Fig. 9.3 MicroRNAs are dysregulated in the context of ischemic stroke. A large number of microRNAs have been implicated in pathogenic mechanisms (excitotoxicity, inflammation, and apoptosis), risk factors, and the outcome of stroke studies. Some of them appear to be potential therapeutic targets (neurogenesis, angiogenesis, and neuroprotection)

24 and 48 h after transient middle cerebral artery (MCA) occlusion, respectively. MiRNAs were discovered to be expressed differently in the normal and ischemic brains, with their pattern of expression changing with the amount of time the brains had been re-perfused. In 2009, a second miRNA profiling study in cerebral ischemia was published. A total of 238 microRNAs were identified in brain samples taken from spontaneously hypertensive rats who had undergone transient MCA occlusion, according to the findings. Several miRNAs were found to be involved, with some having upregulated expression and others having downregulated expression at 3, 6, 12, 24, and 72 h following reperfusion. Further data to access the effect of global ischemia in rat hippocampi suggested the involvement of miRNAs in global ischemia. Like the preceding results, a number of miRNAs were significantly dysregulated after transient global ischemia and 30 min of reperfusion (Fig. 9.3).

9.7 MicroRNAs and Blood-Brain Barrier

Studies in rodent models of ischemic stroke have suggested that microRNAs influence blood-brain barrier function. The majority of miRNAs, such as miR-155, miR-150, miR-210, miR-143, miR-130a, and miR-15a/16-1, have been shown to negatively regulate BBB permeability (Bai et al. 2018; Caballero-Garrido et al.

2015; Fang et al. 2016; Ma et al. 2017; Yao et al. 2018; Zuo et al. 2019). These microRNAs have the potential to increase ischemia-induced BBB leakage by targeting tight junctions either directly or indirectly. According to one study, in a model of distal MCAO, mice treated with miR-155 inhibitors had improved microvascular integrity in the peri-infarct area, whereas controls did not (Caballero-Garrido et al. 2015). Furthermore, a luciferases reporter assay revealed that claudin-1 is a direct target of miR-155, which was previously unknown. Inhibition of miR-155 also resulted in an increase in the interaction between claudin-1 and ZO-1, as well as an indirect upregulation of ZO-1 expression. MiR-15a/16-1 has been shown to impair BBB integrity by directly binding to the 3′-UTR of claudin-5, and endothelial cell-specific deletion of the miR-15a/16-1 cluster (Zuo et al. 2019). Claudin-5 is taken into consideration because it is the claudin that is found in the greatest abundance in endothelial cell-to-cell junctions (Nitta et al. 2003). Furthermore, miR-320 has been linked to cerebral edema because it suppresses the expression of aquaporins 1 and 4, which are required for water reabsorption. An increase in miR-320 levels following a stroke has been observed (Sepramaniam et al. 2010). A recent study in hypoxic mouse and human brain microvascular endothelial cells identifies miR-212/132 as key players in the hypoxic BBB (Burek et al. 2019). The authors identified three miR-212/132 targets that have been implicated in the regulation of barrier properties at the BBB. In conclusion, this study showed that the miR-212/132 family contributes to compromised barrier properties in hypoxic human brain microvascular endothelial cells via a mechanism involving Cldn1, Jam-C, and Tjap1 regulation. In contrast, when mice with an endothelial cell-selective deletion of miR-15a/16-1 were subjected to an experimentally induced stroke, their brain infarcts were smaller, their BBB leakage was less, and their peripheral immune cell infiltration was less (Ma et al. 2020).

Bevan et al. (2012) suggest that identifying genetic variation in a particular miRNA may be a viable strategy for predicting future stroke risk in certain populations (Du et al. 2017). Finally, the authors demonstrated the importance of genetic variation in stroke, which varies depending on the type of stroke: 16.1% for small-vessel disease, 32.6% for cardioembolic disease, and 40.3% for large-vessel disease, among others (Bevan et al. 2012). A growing body of evidence points to the influence of miRNA single nucleotide polymorphisms (SNPs) on miRNA functionality, as stated by Du et al. (2017). SNPs, because they are inherited genetic variations, are useful for predicting future cerebrovascular events. In a meta-analysis encompassing 3372 stroke patients and 4394 controls, the author found that miR-149 SNPs are associated with an elevated risk of future stroke.

Following a stroke, neurogenesis, angiogenesis, apoptosis, and synaptic plasticity occur (Raza et al. 2018). A number of findings have established the role of microRNAs in the aforementioned restorative processes. For example, Stary et al. (2015) demonstrated the role of miR-200c in the post-stroke brain by enhancing neuronal migration and synaptogenesis. Changes in miR-200c levels in the brain were found to be inversely related to reelin, a regulator of neuronal migration and synaptogenesis. It was discovered that inhibiting miR-200c led to an increase in cell survival after in vitro oxidative injury (Lee et al. 2012a, b). These findings show that

miR-200c promotes brain cell death by inhibiting reelin expression, and lowering post-stroke miR-200c may be a potential therapeutic target for treating the long-term effects of stroke. Contrastingly, miR-134 regulates synaptic spine volume in a negative manner. Notably, Arc is a key modulator of synaptic plasticity (Shepherd and Bear 2011) and has been found in stroke brain (Donath et al. 2016; Kadam et al. 2010). Following a stroke, Arc expression is reduced in the infarct core but increased in the peri-infarct cortex. A number of miRNAs regulate Arc expression (Gong et al. 2019; Bo et al. 2014). Ectopic expression of miR-34a, miR-193a, or miR-326 downregulates endogenous Arc protein expression in response to brain-derived neurotrophic factor (BDNF) treatment (Wibrand et al. 2010). Cell-penetrating, peptide nucleic acid inhibitors of miR-326 administration increased Arc mRNA expression and synaptic plasticity. In ischemic stroke patients, angiogenesis has occurred in post-stroke penumbra, and the number of new vessels correlates with longer survival miR-210 helps regulate angiogenesis in response to ischemic brain injury. For example, miR-210 participation (upregulation) can activate the Notch signaling pathway, which may contribute to angiogenesis following stroke (Kulshreshtha et al. 2007; Crosby et al. 2009). After ischemia, damaged neurons, activated microglia, and inflammatory cells emit pro-inflammatory cytokines and other potentially deadly chemicals, causing the blood-brain barrier (BBB) to break down, resulting in edema and cerebral injury. Likewise, miR-424 is intended to assist stroke patients by blocking microglia activation by inhibiting transition factors such as CDC25A, CCND1, and CDK6 (Zhao et al. 2013). miR-181c has been shown to reduce TNF-alpha levels after ischemia, thereby preventing neuronal death (Zhang et al. 2015). To study the long-term impact and putative molecular pathways of endothelial miR-15a/16-1 cluster on cerebral ischemia-induced angiogenesis, an endothelium-targeted miR-15a/16-1 conditional knockout (EC-miR-15a/16-1 cKO) animal model was generated. The findings show that EC-targeted deletion of the miR-15a/16-1 cluster improves long-term neurological outcomes following cerebral ischemia by enhancing angiogenesis in the penumbral regions. The study further revealed that VEGFA (vascular endothelial growth factor), VEGFR2 (VEGF receptor 2), FGF2 (fibroblast growth factor 2), and FGFR1 (fibroblast growth factor receptor 1) are all direct downstream targets of miR-15a/16-1 translational suppression. Furthermore, the other studies indicated that the loss of miR-15a/16-1 activity in the vascular endothelium aids post-stroke angiogenesis and long-term neurological rehabilitation (Sun et al. 2020). It has been demonstrated that overexpression of miR-15a can suppress post-stroke angiogenesis by inhibiting the expression of proangiogenic factors in the brain (VEGF, FGF). As a result, decreasing the levels of miR-15a can lead to an increase in post-stroke angiogenic activity (Yin et al. 2010).

Cell death occurs during a stroke as a result of the depletion of oxygen and glucose in neurons. The primary mechanisms of cell death are necrosis and apoptosis. Apoptosis occurs in the penumbra of the infarct, whereas necrosis occurs in the core. The miRNAs involvement in stroke has been documented in numerous findings. The neurotrophin receptor p75 (NTR) has been linked to stroke-induced apoptosis. miR-592 regulates the expression of p75 (NTR). The levels of miR-592

are inversely related to the levels of p75 (NTR). The expression of p75 (NTR) increases following ischemia, while the expression of miR-592 declines following the same condition (Irmady et al. 2014). While studying the effect of exosomes derived from miR-146a-5p-enriched bone marrow mesenchymal stem cells (BMSCs-miR-146a-5p-Exos) on experimental intracerebral hemorrhage, Duan et al. (2020) demonstrated that miR-146a-5p-enriched BMSCs-Exos could provide neuroprotection and functional improvements following intracerebral hemorrhage by inhibiting neuronal apoptosis and inflammation associated with the inhibition of microglial M1 polarization by downregulating the expression of IRAK1 and NFAT5. Further MiR-21 overexpression has also been shown to be neuroprotective. This is due to a decrease in the expression of Fas ligand, which is a cell death inducer. By inhibiting the translation of Bcl-2, miR-15a has been shown to reduce its levels. Consistent with the above findings, peroxisome proliferator-activated receptor (PPAR)-delta regulates miR-15a expression, and PPAR-delta inhibits pro-apoptotic miR-15a, thereby provides neuroprotection (Yang et al. 2017). Taken together, the findings suggest that miRNA plays a regulatory role in stroke via participating in a variety of neurorestorative mechanisms.

9.8 MiRNA in Preconditioning-Induced Neuroprotection in Stroke

Preconditioning is a phenomena in which a low dose of an otherwise damaging stimulus induces tolerance to a subsequent detrimental event such as observed in stroke (Dirnagl et al. 2009; Lee et al. 2010). To date, studies on miRNA changes induced by preconditioning have identified numerous miRNAs that change expression levels after preconditioning. Approximately 40% of miRNAs that were changed in the same direction by preconditioning were observed in more than one study. Besides hypoxia, chemical priming and even miRNAs are some of the preconditioning stimuli that can protect the brain from ischemic injury.

In the above lines, Lee et al. (2010) investigated miRNA profiles after ischemic preconditioning and the associated neuroprotective mechanisms. In the early stages of ischemic preconditioning, the expression of two microRNA families, miR-200 and miR-182, was found to be increased. The miR-200 family of microRNAs was discovered to be neuroprotective by downregulating the levels of prolyl hydroxylase 2. Vartanian et al. (2015) demonstrated that CpG preconditioning regulates miRNA expression by altering the process of genomic reprogramming in the cell. Using a microarray, the authors investigated the expression of microRNAs in the brain in response to CpG preconditioning before and after ischemic stroke. A number of neuroprotective genes were found to be upregulated in animals who had received either CpG or vehicle treatment following a stroke, indicating that miRNAs are involved in genomic reprogramming to improve neuroprotection. Of note, the suppression of the 22 differential miRNAs was associated with an increase in the expression of 16 predicted gene targets. A similar study found that ischemic preconditioning regulates the expression of microRNAs as well as a predicted target,

methyl-CpG binding protein 2 (MeCP2), in the mouse cortex. The authors revealed that miRNA expression varies consistently within each group. Preconditioning-controlled miRNAs are primarily predicted to target mRNAs that encode transcriptional regulators, with MeCP2 being the most prominent. Preconditioned cortex lowers the levels of miR-132, which regulates MeCP2 expression. Preconditioning causes a quick increase in MeCP2 protein, but not its mRNA, in the mouse cortex (Lusardi et al. 2010). These findings suggest that ischemia preconditioning may regulate miRNA expression in order to activate neuroprotection-associated signaling pathways in the event of ischemia, thereby reducing ischemic injury to the brain following stroke.

9.9 MiRNA-Based Therapeutics for Stroke

Multiple studies have investigated the therapeutic values of miRNA mimics in stroke. miRNA mimics protect against ischemic injury in animal stroke models including miR-29b, miR-223, miR-29c, miR-17-92, miR-124, miR-210, miR-23a-3p, miR-139-5p, miR-99a, miR-107, miR-207, miR335, miR-22, miR-9, miR-378, miR-122, miR-210, miR-455, miR-93, and miR-363 (Sun et al. 2018). The above miR mimic reduces infarct volume and edema. Prior to MCAO, miR-29b or miR-29c mimic delivery reduced stroke-induced infarction, edema, and BBB disruption while improving functional recovery (Wang et al. 2015; Khanna et al. 2013; Pandi et al. 2013). Huang et al. (2018) found that the miR-210-LNA therapy had a long-lasting inhibitory effect on miR-210 and inhibited MCAO-increased miR-210 in the ipsilateral hemisphere for up to 48 h after MCAO. This is consistent with recent findings that miR-16 antagomir's inhibitory effect on miR-16 expression continued for at least three days following intracerebral injection (Krutzfeldt et al. 2007). Importantly, the current work found that inhibiting miR-210 reduced acute inflammatory reactions and decreased the expression of pro-inflammatory cytokines and chemokines up to 24 h after MCAO, suggesting that miR-210 blocking may have a neuroprotective effect. When given with the miR-17-92 cluster antagomir, the functional outcome and neuronal plasticity of MCAO rats improved. Finally, inhibiting the miR-17-92 cluster increases NPC proliferation, differentiation, and survival in the subventricular zone by targeting phosphatase and tensin homolog (Liu and Wang 2016; Xin 2017). Furthermore, in a rat neonatal hypoxia-ischemia model, ICV injection of a miR-139-5p mimic reduced infarct volume and inhibited neuronal apoptosis. The miR-107 mimic decreased ischemic brain infarction and increased the number of capillaries in the penumbral area, most likely by increasing endothelial VEGF165/164 levels to promote angiogenesis (Li and Zhang 2015). By reducing microglial and caspase-3 activation, a Let-7c-5p mimic intracerebrovascular injection reduced infarct volume and mitigated neurological impairments (Ni et al. 2015). In the study of intraventricular administration of miR-107 agomir, a rodent model of permanent ischemic stroke, the treatment promoted VEGF mRNA and protein expression, promoting brain vascularity and increasing capillary density in the ischemic boundary zone (Zhu 2015). In addition,

intravenous administration of miR-15a/16-1 antagomir reduced the volume of the cerebral infarct and reduced brain water content, while also increasing anti-apoptotic proteins and decreasing pro-inflammatory molecules (Yang et al. 2017).

9.10 MicroRNAs Mediated Neurogenesis in Stroke

As we learn how to control and manipulate cell-specific miRNAs, therapies for tissue repair are becoming promising (Juranek et al. 2013). However, due to potential delivery and off-target effects, using miRNAs as therapeutic targets still poses numerous challenges. MicroRNAs are critical regulators of adult neurogenesis (Liu et al. 2011). Observations suggest that NSCs also express miRNAs (Barca-Mayo and Lu 2012; Kawahara et al. 2012; Dugas and Notterpek 2011; Junker et al. 2011). For instance, miR-124, the most abundant neuronal miRNA, is expressed in the SVZ of adult rodent brains (Akerblom et al. 2012; Cheng et al. 2009). Endogenous miR-124 deficiency in neural progenitor cells can result in the loss of neuronal differentiation, whereas miR-124 overexpression can promote neuronal differentiation in the mouse brain (Akerblom et al. 2012, Cheng et al. 2009). Sox9, an SRY-box transcription factor, is a physiologic target of miR124. Furthermore, adult SVZ neural progenitor cells express miR-9. miR-9 negatively regulates neural stem cell proliferation and differentiation by inhibiting the expression of the orphan nuclear receptor TLX (Zhao et al. 2009). miRNAs function like rheostats, finely tuning many biological processes, such as angiogenesis, inflammation, hypoxia response, and stem cell biology, by influencing gene regulation (Sen 2011). It is possible that cell-based therapies will be beneficial in regulating miRNA expression (Juranek et al. 2013). The release of microvesicles by cells delivered via intravenous injection stimulates the expression and release of miRNA by endogenous brain cells, resulting in neurorestorative effects after stroke (Juranek et al. 2013).

On the above lines, Lee et al. (2012a, b) demonstrated for the first time that miR-206 modulates BDNF as well as an antagomir miR-206 (a miR-206 inhibitor) in Tg2576 mice. To brief, miR-206 inhibition improved BDNF levels and memory performance, as well as hippocampal synaptic density and neurogenesis. Cultured neural progenitor cells derived from the SVZ of animals with cerebral ischemia altered the miRNA expression profiles associated with non-ischemic SVZ neural progenitor cells, according to Liu et al. (2013). Notably, the neuron-specific miRNA miR-124a was found to be significantly reduced in ischemic SVZ NPCs from a rat MCAO model. Jagged-1 and Notch signaling, both well-known neurogenesis regulators, were found to be potential targets of miR-124 in NSCs (Liu et al. 2011). It was also discovered that a specific miR-17-92 cluster is elevated in ischemic NPCs. NPCs overexpressing the miR-17-92 cluster proliferated and survived due to inhibiting PTEN (phosphatase and tensin homolog), whereas Shh stimulates N-myc to stimulate miR-17-92 cluster expression in NPCs (Liu et al. 2013). Pan et al. (2019) used a transgenic mouse line with conditional ablation of the miR-17-92 cluster in nestin lineage NSCs to demonstrate that ablation of the miR-17-92 cluster significantly reduced the number of proliferating NSCs and

neuroblasts, and impaired hippocampal-dependent learning and memory. In further analysis, bioinformatics analysis of 18 and 21 miRNAs found that these miRNAs influence several signaling pathways, including transforming growth factor-A, Wnt, and Sonic hedgehog (Shh) signals, all of which are known to regulate neural stem cell function.

9.11 Conclusions

It is now well established that miRNAs play a critical role in the proliferation and differentiation of neural cells and that their dysregulation may result in neurodegeneration. We described numerous processes that occur after a stroke and explored the role of microRNAs in influencing these processes in this chapter. Thus, this snapshot of miRNA action and dysregulation captures the sophisticated regulatory network involved in stroke pathophysiology. We believe, further elucidation of the complexities of these micro-regulators will elucidate novel developmental pathways and generate novel strategies for promoting neurorestoration after a stroke.

9.12 MicroRNA in Neurogenesis Post Stroke

S. no.	MiRNA	Outcome	Reference
1.	microRNA-210-LNA	1. Decreased cerebral infarction and ameliorated behavioral deficits	Huang et al. (2018)
		2. Long-term behavioral recovery	
		3.Macrophage infiltration and microglial activation in the brain were inhibited	
2.	miR-9	1. Enhanced neuronal survival and regeneration	Nampoothiri and Rajanikant (2019)
3.	miR-497	1. Promoted ischemic neuronal survival through upregulating anti-apoptotic protein, bcl-2	Sinoy et al. (2017)
4.	miR-98	1. Locomotor impairment reduced	Bernstein et al. (2020)
5.	MicroRNA-124	1. Enhanced survival and neuronal differentiation of neural stem cells	Saraiva et al. (2018)
		2. Decreased neuronal cell death	
6.	miR-19a-3p	1. miR-19a-3p mediates cerebral ischemic injury by targeting ADIPOR2	Ge et al. (2019)
7.	miR-27b	1. Enhances neurogenesis via AMPK activation	Wang et al. (2019)
8.	miR-126	1. Improved functional recovery	Geng et al. (2019)
		2. Enhanced neurogenesis	
		3. Inhibited neuroinflammation	

(continued)

S. no.	MiRNA	Outcome	Reference
9.	miR-7-2-3p and miR-1908	1. Circulating miRNAs in sera could be potential novel risk factors for ischemic stroke	Gui et al. (2019)
10.	miR-140-5p, miR-221-3p and miR-140-5p	1. miR-140-5p is involved in the pathogenesis post-stroke depression	Liang et al. (2019)

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Exosomes as a Diagnostic Tool and Stem Cells' Exosomes as a Promising Cell-Based Cell-Free Therapeutic Tool for Ischemic Stroke

10

Ahmed Abdelfattah-Hassan and Doaa Ibrahim

Abstract

Ischemic stroke is a leading disease that is responsible for deaths and long-term disability all over the world. Intense research has been conducted to find suitable therapeutics and, so far, this quest showed no success. Current therapeutic approaches cover only two concepts, elimination of the cause of ischemia (thrombolysis or thrombectomy) and/or the rehabilitation to try to restore affected functions. No medication has shown promise in the prevention, reduction, or repair of damaged neural cells and the associated neurological functions. In some cases, a limited endogenous restoration of function has been seen following stroke in the brain, and stem cells-based therapeutic application has been shown to efficiently enhance this endogenous brain repair and improve the restoration of affected functions. Exosomes are nanosized vesicles that are produced by most body cells and carry beneficial cargo to transport them between cells. The focus of this book chapter is on the use of exosomes as potential diagnostic tools (containing various important markers) for ischemic stroke and, more specifically, on the recent interest in stem cells-derived exosomes as a promising therapeutic option for efficient neurorestoration following stroke.

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Keywords

Extracellular vesicles · Cell-free therapy · Neuronal healing · Neuroprotection · Angiogenesis · Post-stroke recovery

10.1 Introduction

Ischemic stroke describes the condition where the blood supply to a part of the brain is suddenly interrupted leading to brain cell's death in the affected area (due to loss of oxygen and nutrients supply) and various degrees of impairment of the functions controlled by the affected area. It remains as one of the leading causes of death and disability in adults throughout the world. It is ranked second in the top 10 most leading causes of death globally, accounting for 11% of the global deaths (WHO Global Health Estimates 2000–2019, https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death, accessed on 7th February, 2021) and ranked third in the global causes of disability-adjusted life year (DALYs, WHO Global Health Estimates during 2019, https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/global-health-estimates-leading-causes-of-dalys, accessed on 7th February, 2021). It is becoming a disease of the young and middle-aged adults more than being a disease of the elderly alone; in addition, males are more susceptible than females (Feigin et al. 2017).

There are several types of stroke, ischemic stroke due to blood clots that obstruct brain blood vessels, hemorrhagic stroke due to rupture of weak blood vessels in the brain (due to high blood pressure, malformations in blood vessels or other causes), transient ischemic attack (TIA or mini-stroke) caused by a temporary clot and is a warning sign predisposing for ischemic stroke, while, lastly, cryptogenic stroke in which the cause can't be determined although blood flow to the brain or part of it is still impaired (American Stroke Association, https://www.stroke.org/en/about-stroke/types-of-stroke, accessed on 8th February, 2021).

Stroke is mainly a disease involving the brain, however, it affects the entire body. Stroke symptoms are variable and include sudden loss of function, the most prominent include loss of speaking, hearing, feeling to the touch or motor functions. The symptoms could include any of sudden numbness or weakness particularly involving one side of the body, also, sudden trouble in speaking or understanding of speech or confusion, sudden visual impairment in one or both eyes; sudden loss of balance or walking disturbance or incoordination, sudden dizziness, or sudden severe headache. Deficit in motor and cognitive functions can be severe and the most commonly noted deficit is hemiplegia (i.e., complete paralysis in one body side). A less severe disability is called hemiparesis (i.e., weakness in one body side), and is less debilitating than hemiplegia. Stroke survivors often have a certain disability that can persist for long time and emotionally affect the recovered patients and, physically or emotionally, prevent them from normal reintegration into the society. Moreover, one in every 4 patients who recover from their first stroke can develop another stroke within 5 years. (National Institute of Neurological Disorders and

Stroke, NINDS, NIH, USA, https://www.ninds.nih.gov/Disorders/All-Disorders/Stroke-Information-Page, accessed on 8th February, 2021).

Ischemic stroke leads to reduced oxygen (hypoxia) and nutrients in affected brain regions thus disturb neuronal metabolism (by altering electron transport, calcium ions, glutamate, arachidonic acid) leading to the destruction of many organelles and, thereby, activating apoptosis and neuronal cell death (Lipton 1999) and causing damage of the functional neurovascular units of the brain (Abeysinghe et al. 2016). As a consequence, inflammatory cytokines are released to activate the local immune responses in the damaged area of the brain and activate microglia, astrocytes, and macrophages (González-Scarano and Baltuch 1999; Prinz and Priller 2010). The release of more inflammatory cytokines can intensify the inflammation in the affected brain areas (Whitney et al. 2009). As a result of cell death, destruction of tight junctions and death of endothelial cells leave the blood-brain barrier (BBB) in an open-state, facilitating the development of edema and the infiltration of more immune cells and, consequently, aggravating the local inflammatory responses (Yang and Rosenberg 2011; Krueger et al. 2015). This multifactorial cellular and molecular response will encourage the formation of glia scar (Adams and Gallo 2018) in an attempt to stop the leakage, close the opened BBB, and activate endogenous repair mechanisms. However, glia scars impede appropriate neurogenesis (Abeysinghe et al. 2016) and therefore the repair of the damaged brain areas is hindered and the impairment of the corresponding brain function is evident.

In general, current stroke treatment can be organized into 3 stages. The first one is preventing the occurrence of stroke by treating predisposing conditions (such as hypertension and diabetes); while this is being applied all over the world it still does not completely prevent the occurrence of stroke. Once acute ischemic stroke occurs, the second treatment stage largely relies on the speed of intervention to remove the formed blood clot, i.e., removing/reducing the ischemia. The third stage focuses on the rehabilitation post-stroke to assist patients who suffered certain disabilities to overcome it. The success rate for the three stages is variable and thus stroke medications are still on the top of the list of most wanted medications. Now, the most common medicines prescribed to prevent/treat ischemic stroke include anti-thrombotic drugs (such as anti-platelet agents and anticoagulants) and thrombolytic drugs which break-up or dissolve the formed blood clots (National Institute of Neurological Disorders and Stroke, NINDS, NIH, USA, https://www.ninds.nih.gov/Disorders/All-Disorders/Stroke-Information-Page, accessed on 8th of February, 2021). While decades of research focused mainly on neural protection and removal of ischemia, the NINDS has named a new main priority in stroke research, which is the treatment of delayed neurorestoration (Chen et al. 2014).

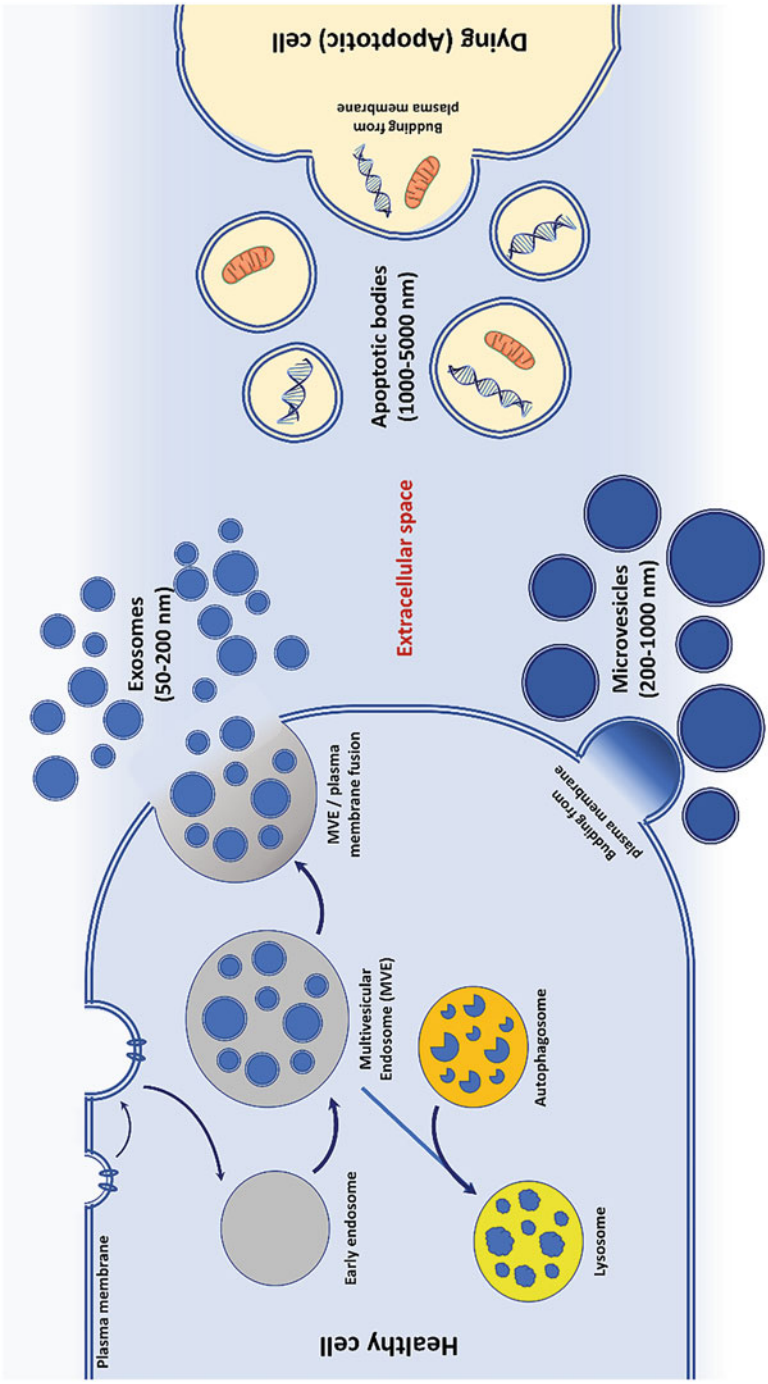

Fig. 10.1 Different types of cell-produced vesicles; exosomes, extracellular vesicles and apoptotic bodies, and their release mechanism

10.2 What Are Exosomes? Their Biogenesis and Functions

Exosomes are one type of a heterogenous population of extracellular vesicles (EVs; Fig. 10.1). The latter are membrane-bound carriers of various intracellular components including proteins, lipids, and different types of nucleic acids (mRNAs, miRNAs, lncRNAs, etc.) (Raposo and Stoorvogel 2013; Yáñez-Mó et al. 2015; Haraszti et al. 2016). It is because of that cargo that EVs have been in the spotlight of research focused on intercellular communication and reprogramming during the last three decades. In addition, EVs' cargo is unique and its composition depends on both the type of cell that produce it and the production method making them good candidates for clinical use as diagnostic/prognostic biomarkers (Li et al. 2017; Liu et al. 2017; Jiang et al. 2019). And, recently, EVs were explored as potential tools for therapeutic and drug-delivery applications (Gao and Jiang 2018; Samanta et al. 2018).

Exosomes are characterized by being the smallest of cell-produced EVs; their size is between 30 and 150 nm in diameter and are surrounded by a membrane similar in structure to the cell's outer membrane. Exosomes are produced inside the cells by the inward budding of the membrane of early endosomes, the latter, after certain maturation steps, form a larger nanovesicles-containing structure known as the multivesicular body (MVB) (Raposo and Stoorvogel 2013). The early endosome itself is also formed by inward budding but from the cell's plasma membrane and, therefore, is involved in internalization of extracellular-milieu components and sorting them inside the cell through various endocytic pathways; which include the endosome-lysosome degradation pathway, slower recycling pathway, or rapid-recycle to the plasma membrane pathway (Naslavsky and Caplan 2018). Thus, some endosomes are not degraded but mature to become MVBs and are, then, sent to the cell membrane to fuse with it and release its various contents, which include the exosomes. The regulation of MVBs fate, exosome formation, and exosomal cargo are still not clearly understood; however, the sphingolipid ceramide and the endosomal sorting complexes required for transport (ESCRT) pathway seem to be implicated to a certain extent (Trajkovic et al. 2008; Wollert and Hurley 2010). Moreover, it has been proposed that a cell can adjust its MVBs content, including exosomal cargo, in response to various factors and depending on the desired response chosen by that cell, however this is still not fully understood (Doyle and Wang 2019).

Exosomes were previously considered as means for cell-waste removal, through which the cells disposed of unwanted broken-down intracellular components (Dalton 1975). However, around 2006, the global scientific community started to perceive exosomes as an important player in intercellular communication (Ratajczak et al. 2006; van Niel et al. 2006). And, this role is facilitated mainly by the transfer of genetic material; including various types of mRNA and miRNAs (Valadi et al. 2007). Exosomes are now shown to be produced from almost all cell types of the body. A representation of exosomes' structure and, so far, identified contents are illustrated in Fig. 10.2.

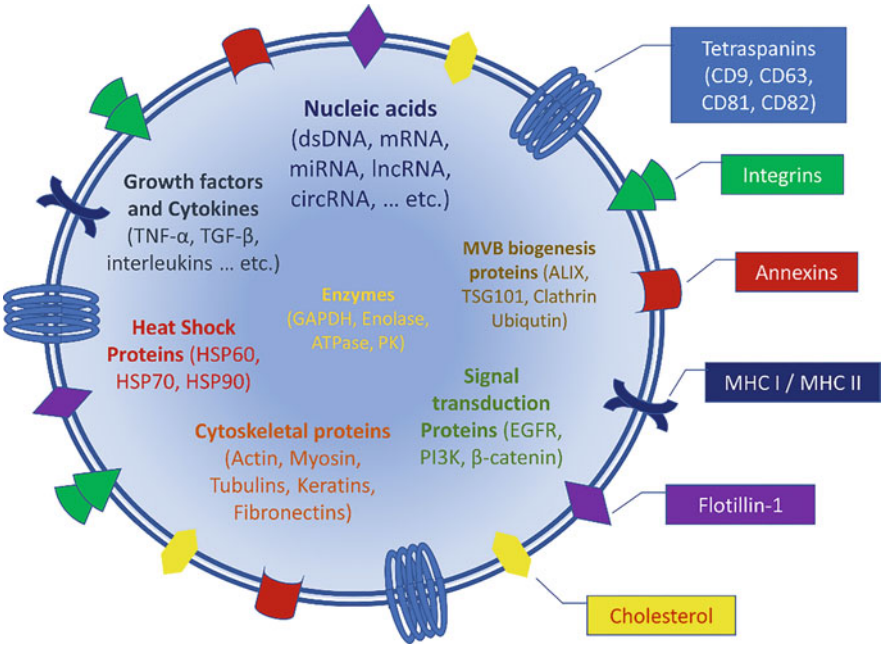

Fig. 10.2 Currently known structure/contents of exosomes

Inside the nervous tissue, exosomes were found to play various roles. They were shown to play important role in the glia-mediated support to neurons, via exosomes secreted by oligodendrocytes (Krämer-Albers et al. 2007; Bakhti et al. 2011). These cultured oligodendrocyte-derived exosomes contained various myelin sheath-promoting factors; including myelin proteolipid proteins, 2′,3′-cyclic nucleotide 3′-phosphohydrolase, myelin basic protein, and myelin oligodendrocyte glycoprotein, in addition to various stress-relieving proteins, which all contribute to proper neuronal functions. Exosomes were also supposed to have normal physiological functions at the synapse; they are thought to be both regulated by neuronal depolarization and regulating the synaptic processes (Fauré et al. 2006; Lachenal et al. 2011). In addition, glia-derived exosomes were found to regulate survival of neurons and their neurite outgrowth, especially in conditions of higher activity or stress, further promoting the idea of glia-neurons interaction through exosomes (Wang et al. 2011).

However, it is not only beneficial factors that exosomes carry, because they were found to propagate inflammatory responses across the blood-brain barrier and within the brain, they also carry proteins associated with brain diseases and thus might contribute to the propagation of these diseases. As, for example, systemic inflammation was found to activate endothelial and other cells of the brain which in turn, via secreted exosomes, further propagated the inflammatory condition to adjacent cells inside the brain. This undesirable effect was demonstrated by examining exosomes and extracellular vesicles obtained from choroid plexus epithelial cells (Balusu et al.

2016), astrocytes (Dickens et al. 2017), microglia (Kumar et al. 2017), and endothelial cells (Couch et al. 2017) following systemic inflammation. Furthermore, exosomes were found to carry infectious prion proteins and contributed to its spread inside the organism, and, thus, these proteins-carrying exosomes were denominated as "infectious exosomes" (Fevrier et al. 2004). They were also associated with the transfer of beta-amyloid peptides and the pathogenesis of Alzheimer's disease (Rajendran et al. 2006), mutated Cu,Zn superoxide dismutase protein in the amyotrophic lateral sclerosis disease (Gomes et al. 2007), α-synuclein in Parkinson's disease pathogenesis (Emmanouilidou et al. 2010), and HIV-1 Tat protein broadening the spectrum of HIV-associated neuronal death (Rahimian and He 2016).

10.3 Exosomes as Biomarkers for Stroke

Early diagnosis of ischemic stroke is vital to assure early treatment and improved prognosis in ischemic stroke patients. Clinical diagnosis of the location and volume of infarction in ischemic stroke is done by computed tomography (CT), magnetic resonance imaging (MRI), and other advanced imaging techniques. However, these methods do not give accurate representation of the cause of the infarction and, therefore, laboratory (hematological and biochemical) testing turn to be very essential but is still challenging.

Recent interest in exosomes has demonstrated that they can be used efficiently as biomarkers for various diseases, since their cargo is disease-specific. And, of the most important components of exosomes, micro RNAs (miRNAs or miRs) play an important role and are constantly being investigated as valid diagnostic and prognostic biomarkers. Plasma exosomes were found to have various types of RNA species, miRNAs are the most abundant (accounting for approx. 76% of exosomal RNA content), while ribosomal RNA, long non-coding RNA, Piwi-interacting RNA, transfer RNA, small nuclear RNA are found with less amounts (9.16, 3.36, 1.31, 1.24, and 0.18%, respectively) together with other types of RNA (Huang et al. 2013). The added benefit of protection to these potential biomarkers, by being enveloped inside exosomes, makes them stable in conditions that otherwise would lead to their destruction. For example, miRNAs were found to be stable in plasma exosomes that were stored under different conditions (Ge et al. 2014).

Within the first 72 h after ischemic stroke, circulating exosomal miR-223 became detectable and was proven to be positively correlated with National Institutes of Health Stroke Scale (NIHSS) scores. In addition, higher expression levels of miR-223 were associated with poor outcomes in ischemic stroke patients (Chen et al. 2017). Within 24 h post-stroke, exosomal miR-134 was positively correlated with NIHSS scores, infarct volume, and poor prognosis of the acute ischemic stroke patients (Zhou et al. 2018). During acute phase ischemic stroke (between days 1 and 3) plasma exosomal miR-422a levels were increased, while in subacute stroke patients (between days 4 and 14), both plasma miR-422a and miR-125b-2-3p were reduced indicating their potential to be used as biomarkers for different ischemic stroke phases (Li et al. 2018a). Also, in acute ischemic stroke patients, serum

exosomal miR-9 and miR-124 (described as brain-specific miRNAs) levels increased and were positively correlated with NIHSS scores, infarct volume, and serum concentration of interleukin 6 (IL-6) (Ji et al. 2016). In addition, the combination of miR-21-5p and miR-30a-5p identified in plasma exosomes was efficient to differentiate between hyperacute phase (within 6 h, of importance is miR-30a-5p), early acute phase (days 1–3), late acute phase (days 4–7), subacute phase (days 8–14), and recovery phase (days >14) in patients with ischemic stroke (Wang et al. 2018b). While, in rats, plasma levels of exosomal miR-122-5p decreased and miR-300-3p increased, and both can serve as potential biomarkers for transient ischemic attack (Li et al. 2018b).

In addition, long non-coding RNAs (lncRNA) and circular RNA (circRNA) within plasma exosomes from ischemic stroke patients can show important variations. For example, lncRNA MIAT, lncRNA H19, and circHECTD1 were all found to be associated with ischemic stroke and could be used as potential biomarkers. LncRNA MIAT, from peripheral blood leukocytes, was found to be increased in ischemic stroke and positively correlated with NIHSS scores, infarct volume, c-reactive protein levels, and bad prognosis in IS patients (Zhu et al. 2018). Also, higher lncRNA H19 levels, in peripheral venous blood samples (most probably containing exosomes), were found in IS patients within 72 h after onset of the disease and polymorphisms in the H19 gene were associated with higher risk of IS development (Rezaei et al. 2021). In addition, expression of plasma (containing exosomes) circRNA was implicated, as circHectd1 was significantly increased with increasing infarct areas, increased neuronal damage and astrocyte activation in transient middle cerebral artery occlusion mouse stroke model and in serum of IS patients (Han et al. 2018). In acute minor stroke, different exosomal lncRNAs were evaluated as potential biomarkers, and it was found that lnc-CRKL-2 and lnc-NTRK3-4 were increased (at least 23 folds), and RPS6KA2-AS1 and lnc-CALM1-7 were decreased (at least 118 times) in acute minor stroke patients (Xu et al. 2020). However, more data is still needed for the validation and clinical use of non-coding RNAs in stroke diagnosis/prognosis.

Similarly, serum miR-223 was shown to be associated with the development of acute ischemic stroke and correlated with NIHSS scores and disease severity (Wang et al. 2014). It was also found that serum miR-223 level was positively correlated with its level in the brain (Wang et al. 2014). Moreover, higher serum miR-21 and lower miR-221 were also shown to be related with ischemic stroke development, and the stroke risk increased with increasing miR-21 or decreased miR-221 serum levels (Tsai et al. 2013). Moreover, plasma miR-21 and miR-24 were positively correlated with each other and both were negatively correlated with NIHSS scores within the first day following stroke caused by acute cerebral infarction (Zhou and Zhang 2014). Furthermore, serum levels of miR-132 were upregulated within one year of first stroke of vascular cause, and it was positively correlated with the severity of post-stroke cognitive impairment (PSCI) in affected patients (Huang et al. 2016). In addition, serum circulating miR-221-3p and miR-382-5p, sampled immediately upon admission to hospital, were found to decrease in ischemic stroke patients compared to healthy individuals (Wang et al. 2017).

Using miRNA microarray analysis from peripheral blood of ischemic stroke patients (from day 1 till 2 years after stroke onset), a panel of 32 miRNAs were highlighted to be unique for the diagnosis of different stroke subtypes (Sepramaniam et al. 2014). Of these 32 miRNAs, five were consistently upregulated during acute stroke irrespective of age, severity, or confounding metabolic complications, these are miR-125b-2, miR-27a, miR-422a, miR-488, and miR-627.

Differences between males and females in the expression of some miRs have to be taken into consideration while using exosomes and their cargo of miRs as diagnostic tools for stroke. A clear example is different levels of miR-23a in ischemic brains of males and females; which was found to contribute to the sex-related differences in the mechanism of stroke-induced cell death (Siegel et al. 2011). This effect was achieved via miR-23a regulation of the X-linked inhibitor of apoptosis (XIAP), which is one of the endogenous caspase-inhibitors, and the inhibition of XIAP aggravated stroke-induced brain damage in females but not in males.

From all this it is clear that various miRNAs and other non-coding RNAs can be used as promising biomarkers for stroke diagnosis/prognosis (Table 10.1). In the near future, with more studies validating panels of these biomarkers and integrating them in one platform will permit fast and accurate diagnosis/prognosis of ischemic stroke.

10.4 Exosome Therapy for Ischemic Stroke Recovery

The current primary treatment of acute ischemic stroke targets mainly the elimination of the ischemic condition via thrombolytic therapy (injection of tissue plasminogen activator, tPA) or thrombectomy (mechanical recanalization of the clogged blood vessels). The time window for applying any of these two approaches is very narrow; only 3–4.5 h from stroke symptoms onset (Zerna et al. 2018; Powers et al. 2019). However, this time window is missed in a large number of cases reducing their validity and can lead to increased bleeding risks and worsened therapeutic outcomes (Emberson et al. 2014; Powers et al. 2019). In addition, clearing the ischemia does not mean that local effects as neuronal cells' damage, oxidative stress, and inflammation are resolved. Therefore, current clinical research focuses more on expanding the treatment time window and preventing or reducing the local damage secondary to ischemia. In this regard, the anti-inflammatory and regenerative effects of stem cells and their secreted factors became of great importance. Therefore, supplying exogenous stem cells or exosomes for the treatment of stroke and other diseases are under heavy investigation, since it is widely accepted that the repairing activity of endogenous stem and progenitor cells is not sufficient to repair damaged brain tissue and restore its normal homeostasis and neurological functions.

Since extracellular vesicles, including exosomes, are vital mediators of cell-to-cell interaction, it is via their content of various important molecules (such as mRNAs, non-coding RNAs, proteins, and other factors) that gene expression and associated biological functions are regulated. Therefore, exosomes derived from

Table 10.1 Exosomes and their cargo as biomarkers for ischemic stroke

Exosomal NcRNA	Exosome Source	Exosome isolation procedure	Model	Increased/ decreased in ischemic stroke	Ref.
Animal models of ischemic stroke					
MiR-126	Serum	Precipitation	MCAO and sham rat model	Transient decrease (3 h post-ischemia)	Chen et al. (2015)
MiR-122-5p	Plasma and CSF	Spin column	MCAO rat model	Decreased (in 10 min TIA model) Its level was correlated with CSF exosomes.	Li et al. (2018b)
MiR-300-3p	Plasma and CSF	Spin column	MCAO rat model	Increased (in 5 min TIA model) Its level was correlated with CSF exosomes.	Li et al. (2018b)
MiR-450b-5p	Plasma and CSF	Spin column	MCAO rat model	Reduced in stroke model. Might play a role in cerebral ischemia pathophysiology.	Luo et al. (2019)
MiR-17-5p MiR-93-5p	Serum and brain	Precipitation, filtration, and sucrose density gradient ultra-centrifugation	SHRS and WKY rat model	Both increased in extracellular vesicles from SHRSP vs. WKY	van Kralingen et al. (2019)
Ischemic stroke patients					
MiR-9 MiR-124	Serum	Precipitation	65 AIS patients and 66 controls	Increased in AIS.	Ji et al. (2016)
MiR-223	Serum	Precipitation	50 AIS patients and 33 controls	Increased in AIS. Positively correlated with NIHSS scores. Its expression was higher in stroke patients with poor outcomes.	Chen et al. (2017)
MiR-21-5p and MiR-30a-5p	Plasma	Spin column	143 IS patients (HIS, AIS, SIS, and RIS) and 24 controls	MiR-21-5p and miRNA-30a-5p together are promising biomarkers for early-stage diagnosing IS and differentiation between different stroke phases.	Wang et al. (2018b)

(continued)

Table 10.1 (continued)

Exosomal NcRNA	Exosome Source	Exosome isolation procedure	Model	Increased/ decreased in ischemic stroke	Ref.
MiR-134	Serum	Precipitation	50 AIS patients and 50 controls	Increased in AIS patients within 24 h after stroke onset compared to control. It correlated with NIHSS scores, infarct volume and positively associated with poor prognosis of stroke patients.	Zhou et al. (2018)
MiR-422a MiR-125b-2-3p	Plasma	Ultra-centrifugation	IS patients	MiR-422a Increased in acute IS. MiR-422a MiR-125b-2-3p were reduced in subacute IS. Potential biomarkers for different stroke phases.	Li et al. (2018a)
MiRNA-17-5p MiRNA -20b-5p	Serum	Precipitation	173 IS patients (48 h post stroke) and 39 controls (stroke mimic)	Both increased in IS compared to stroke mimics. Their expression differed by stroke subtype.	van Kralingen et al. (2019)
MiR-27b-3p	Serum	Precipitation	173 IS patients and 39 stroke mimics	miR-27b- 3p was increased in IS compared to stroke mimic, and it's higher in SVD than other types of stroke.	van Kralingen et al. (2019)
MiR-27b-3p	Plasma	Spin Column	19 IPH, 17 SAH and 21 IS patients	High level of exosomal miR-27b-3p in ischemic stroke compared to hemorrhagic stroke types. It can be used in differentiating between ischemic and hemorrhagic stroke subtypes.	Kalani et al. (2020)

(continued)

Table 10.1 (continued)

Exosomal NcRNA	Exosome Source	Exosome isolation procedure	Model	Increased/ decreased in ischemic stroke	Ref.
LncRNA MIAT	No exosomes	Isolation of peripheral blood leukocytes	IS patients	Increased in IS. Positively correlated with NIHSS scores, infarct volume, c-reactive protein levels, and bad prognosis in IS patients	Zhu et al. (2018)
lncRNA H19	No exosomes	Peripheral venous blood samples	IS patients	Higher levels were found in IS patients within 72 h after IS onset	Rezaei et al. (2021)
circHectd1	No exosomes	Plasma from AIS patients and brain tissue from MCAO mice	MCAO mouse model and AIS patients	Levels increased with increasing infarct areas, increased neuronal damage and astrocyte activation	Han et al. (2018)
lnc-CRKL-2 lnc-NTRK3-4	Serum	Precipitation	100 AMS patients and 100 controls	Exosomal lnc-CRKL-2 and lnc-NTRK3-4 are increased in AMS patients.	Xu et al. (2020)
RPS6KA2-AS1 lnc-CALM1-7	Serum	Precipitation	100 AMS patients and 100 controls	Exosomal RPS6KA2-AS1 and lnc-CALM1-7 are decreased.	Xu et al. (2020)
Other stroke types					
MiR-193b-3p	Plasma	Filtration and ultra-centrifugation	SAH mouse model	Exosomal miR-193b-3p is increased in subarachnoid hemorrhage (SAH) mice compared to the control sham mice model.	Lai et al. (2020)
hsa-miR-486-3p	Plasma	Filtration and ultra-centrifugation	13 SAH patients and 13 controls	Hsa-miR-486-3p is increased in the experimental group (24 h post-SAH) compared to healthy control.	Lai et al. (2020)

(continued)

Table 10.1 (continued)

Exosomal NcRNA	Exosome Source	Exosome isolation procedure	Model	Increased/ decreased in ischemic stroke	Ref.
MiR-193b-3p	Plasma	Filtration and ultra-centrifugation	13 SAH patients and 13 controls	Exosomal hsa-miR193b-3p is increased in the experimental group (24 h post-SAH) compared to healthy control	Lai et al. (2020)
hsa-miR-369-3p hsa-miR-410-3p	Plasma	Filtration and ultra-centrifugation	13 SAH patients and 13 controls	Exosomal hsa-miR-369-3p and hsa-miR-410-3p are decreased in the experimental group (24 h post-SAH) compared to healthy control.	Lai et al. (2020)
MiR-630	CSF	Precipitation	8 aSAH patients and 4 control patients	Exosomal miR-630 showed lower level in SAH than in control patients.	Sun et al. (2019)
MiR-27b-3p	Plasma	Spin Column	60 patients with ACAS	Exosomal miR-27b- 3p is increased in patients with ACAS progression.	Dolz et al. (2017)
MiR-199b-3p MiR-130a-3p MiR-221-3p MiR-24-3p	Plasma	Spin Column	60 patients with ACAS	Exosomal MiR-199b-3p, MiR-130a-3p, MiR-221-3p, and MiR-24-3p are increased in patients with ACAS progression.	Dolz et al. (2017)

cells with therapeutic potential, as mesenchymal stem/stromal cells (MSCs), can be of important therapeutic value compared to cell-based therapies which face various obstacles in the present time (Fig. 10.3). Taking into account that MSCs have been extensively investigated for their therapeutic potential in various diseases via their transdifferentiation to replace damaged cells. Nevertheless, recent evidence suggested that the beneficial effects of MSCs are rather attributed to their secreted bioactive molecules within extracellular vesicles and exosomes. In fact, MSCs exosomes were found to be as efficient as MSCs transplantation when used in various disease models (Rani et al. 2015; Doeppner et al. 2015; Marote et al. 2016). These therapeutic effects were specific to MSCs-exosomes and not related to, for example, dermal fibroblasts-derived exosomes (Gatti et al. 2011).

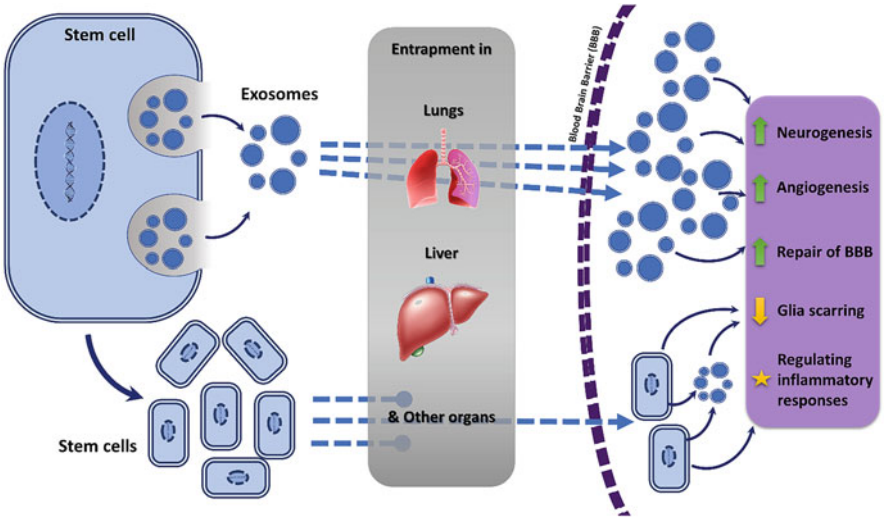

Fig. 10.3 Comparison between stem cells and stem cell-derived exosomes in the therapy of ischemic stroke

Exosomes isolated from MSCs have been investigated for their therapeutic potential in animal models of IS and other neurodegenerative diseases (Xin et al. 2014; Zagrean et al. 2018). Proteomic analysis of exosomes obtained from MSCs showed that these nanovesicles harbor more than 2400 proteins that are associated with various cellular processes, and that they initiate poststroke brain repair with a single dose (Otero-Ortega et al. 2017). As will be discussed here in, exosomes' beneficial effects in treating ischemic stroke are a combination of neuroprotection, neurogenesis, angiogenesis, and immune modulation, that overall contribute to neurological functional improvements (Fig. 10.3).

10.4.1 Direct Beneficial Effects on Ischemic Areas

10.4.1.1 Exosomes for Improved Neurogenesis and Reduced Glia Scaring

One of the most important aspects of ischemic stroke treatment is the preservation and repair or regeneration of damaged nerve cells and affected areas of the brain. Still, current clinical approaches for ischemic stroke are only focused on thrombolytic therapy or thrombectomy, and there are no clinically available therapies for neurogenesis and, hence, stem cells and their secreted extracellular vesicles come as strong candidates. Other than neurons, glia cells will also be activated following ischemic stroke and the resulting functional and morphological changes in glia cells (known as glia scar) also interferes with neurogenesis and delay stroke recovery (Abeysinghe et al. 2016). However, completely abolishing glia scarring response leads to worse outcomes, such as increased infarct size and reduced functional

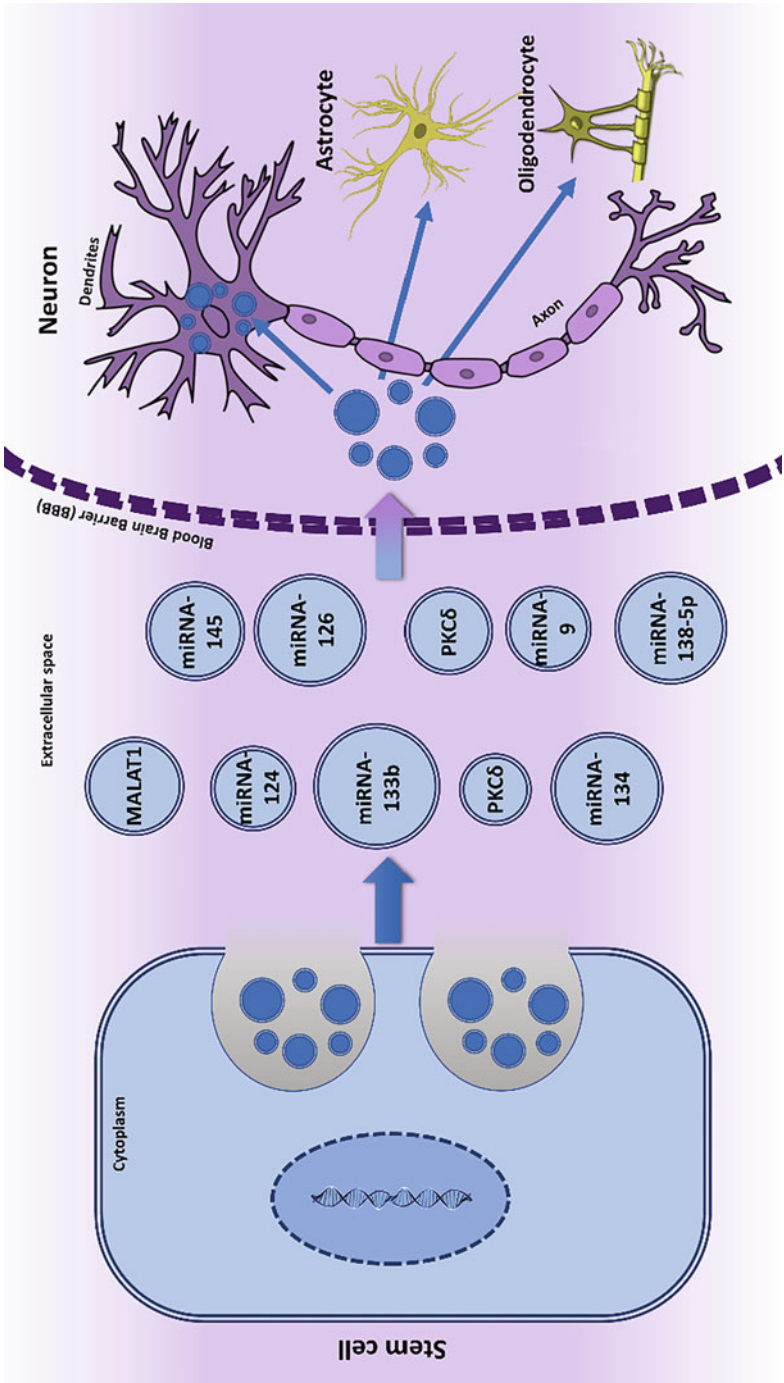

Fig. 10.4 Effects of MSCs produced exosomes on neuronal regeneration and repair following stroke

recovery following stroke (Li et al. 2008; Liu et al. 2014), and it is more appropriate to target the modulation of glia scarring responses than completely abolishing them. Therefore, research is still in progress to achieve both improved neurogenesis and reduced glia scarring following stroke, exosome therapy can be a good approach (Fig. 10.4).

In a rat model of ischemic stroke, MSCs were found to promote functional recovery (evident around 14 days post-stroke and post-administration), neurite outgrowth (neurite branch number and total neurite length), and reduced glial scarring via exosome-mediated transfer of miR-133b to injured areas of the brain (Xin et al. 2012). In the latter study, when *in vitro* cultured MSCs were subjected to extracts from ischemic brain tissues, miR-133b was produced in MSCs and their exosomes. This was further confirmed when exosomes derived from MSCs with upregulated miR-133b increased neurite outgrowth and those derived from MSCs with downregulated miR-133b reduced it when compared to exosomes from naïve MSCs (Xin et al. 2013b). The mechanism of miR-133b action was investigated *in vitro*, it was found to promote neurite outgrowth in PC12 cells (a mature neural cell model of neurite outgrowth) and encouraged axonal regeneration in cultured primary cortical neurons; this was achieved via suppressing RhoA (Ras Homolog Family Member A) and activating PI3K/Akt and ERK1/2 pathways (Lu et al. 2015). Also, exosomal transfer of miR-134 to *in vitro* injured oligodendrocytes reduced the expression of caspase-8 and thus reduced their apoptosis (Xiao et al. 2019), this should preserve oligodendrocytes' functions *in vivo* and prevent further damage to the nerve cells in ischemic areas. Similar effects were noted *in vitro* with astrocytes after exosomal transfer of miR-138-5p which promoted astrocytes proliferation and migration and reduced neuronal damage *in vivo* in a mouse model of ischemic stroke (Deng et al. 2019).

Following induction of stroke in a rat model, exosomes from MSCs were administered after 24 h and were found to enhance functional recovery following stroke (Xin et al. 2013a). This was achieved through increasing axonal density (indicating neurite remodeling), synaptophysin positive cells (as a marker of synaptogenesis), and increased the number of migrating neuroblasts along the boundaries of the ischemic areas in the brain.

In an *in vitro* model of neuronal injury, immortalized mouse hippocampal cells (HT22) were treated with exosomes derived from human adipose MSCs (hAD-MSCs) which resulted in activation of protein kinase C δ (PKCδ) which in turn promoted the survival and proliferation of injured neurons (El Bassit et al. 2016). This pro-survival effect was mainly mediated through the long noncoding RNA MALAT1 transferred inside exosomes derived from hAD-MSCs, while insulin addition further enhanced this effect.

Since diabetes is a predisposing factor to stroke, exosomes from bone marrow MSCs (BM-MSCs) or using umbilical cord blood MSCs (UCB-MSCs) following induction of stroke in diabetic mice also showed improvements in neurological functions and reduction in the brain damage following stroke (Chen et al. 2016a; Cui et al. 2016). In addition, exosomes produced by BM-MSCs isolated from diabetic mice (type 1 or type 2) showed superior results in terms of neuroprotection,

white matter remodeling and reduced vascular damage in a stroke model in diabetic mice compared to exosomes isolated from normal MSCs (Cui et al. 2016; Venkat et al. 2020). These effects were mediated through the roles of miR-9, miR-126, and miR-145/ABCA1/IGFR1 pathway on vascular and white matter remodeling in ischemic diabetic mice (Chen et al. 2016a; Cui et al. 2016; Venkat et al. 2020).

Moreover, administration of exosomes derived from xenogenic AD-MSCs of mini-pigs was shown to exert neurological improvements in a rat model of ischemic stroke (Chen et al. 2016b). Xenogenic AD-MSCs exosomes similar to AD-MSCs administration led to reduction in the protein expression of inflammatory, oxidative stress, fibrotic, apoptotic, and brain damage markers in the brain-infarct zone; all this should contribute to preserving the brain tissue and improving the neurological functions poststroke.

Exosomes isolated from human BM-MSCs were shown to increase numbers of immature and mature neurons at the site of injury in a mouse model of ischemic stroke (Doeppner et al. 2015), this led to improvement in motor coordination and nurturing long-term neuroprotection poststroke.

In a model of subcortical infarction, exosomes obtained from allogenic AD-MSCs showed functional improvements that were associated with axonal sprouting and growth, oligodendrocyte formation, tract connectivity and white matter repair and remyelination (Otero-Ortega et al. 2017). In addition, cortical neurogenesis was achieved following ischemic stroke when miR-124 was delivered in modified exosomes that specifically target neurons (Yang et al. 2017). This was confirmed by the reduced expression of neural progenitor markers SOX2 and Nestin and increased expression of immature neuronal marker doublecortin (DCX) in the ischemic cortex; suggesting the initiation of neural differentiation of these progenitors. Of note, miR-124 is the most brain-enriched miRNA and it is an important determinant of the fate of neural progenitor cells in the subventricular zone (SVZ) of the brain (Åkerblom et al. 2012), and it acts via Notch signaling pathway to regulate the proliferation of neural progenitor cells in the SVZ following stroke (Liu et al. 2011).

10.4.1.2 Exosomes for Improved Angiogenesis Following Stroke

Angiogenesis is an essential step for the recovery of ischemic areas of the brain and the regain of lost neurological functions. In this regard, MSCs-derived exosomes were found to increase the number of von Willebrand stained cells (a marker for endothelial cells) at the site of injury in a rat model of ischemic stroke (Xin et al. 2013b). Similarly, exosomes from BM-MSCs were shown to increase the numbers of $CD31^+$ labeled endothelial cells at the site of injury (Doeppner et al. 2015). Also, exosomes produced by BM-MSCs isolated from diabetic mice increased capillary tube formation in ischemic areas of the brain (Cui et al. 2016).

Local micro-angiogenesis was improved in the ischemic areas of the brain in a mouse model, after 14 days poststroke, following administration of BMS-exosomes loaded with miR-210 (Zhang et al. 2019). This led to improved endothelial cells proliferation, promoted micro-angiogenesis, and increased the survival rate of ischemic mice receiving miR-210 loaded exosomes.

Regardless of the available information which confirms the angiogenic benefits of MSCs-exosomes, the exact mechanisms by which they exert these long-term (i.e., noticed for at least 2 weeks after a single exosome injection) proangiogenic properties is still largely unknown.

10.4.1.3 Exosomes for Repair of Blood-Brain Barrier (BBB)

Loss of BBB integrity is one of the important sequels of ischemic stroke, which is predominantly caused by the degeneration of endothelial cells and loss of tight and adherens junctions and thus lead to breaking down the BBB, which holds a threat for cerebral bleeding and hemorrhage while performing therapeutic recanalization (Yang and Rosenberg 2011; Krueger et al. 2015). In addition, the damaged BBB permits a free pass for immune cells that infiltrate the infarct areas and, thus, aggravating the local inflammatory responses leading to less efficient repair of the damaged areas of the brain (Whitney et al. 2009). Therefore, in treating ischemic stroke, it is essential to target the repair of damaged BBB as well.

Similar to neurological outcomes of exosomes produced by BM-MSCs isolated from diabetic mice, exosome administration led to reduced vascular damage in the ischemic brain areas (Cui et al. 2016). Also, xenogenic AD-MSCs exosomes led to reduction in the protein expression of aquaporin-4 (a marker of brain edema) suggesting their protective effect on BBB in the brain-infarct zone (Chen et al. 2016b). In addition, the administration of exosomes obtained from MSCs of diabetic mice increased tight junction protein (ZO-1) and thus contributed to improving the integrity of BBB (Venkat et al. 2020). Furthermore, the extracellular vesicles (containing exosomes) isolated from human BM-MSCs, by ultracentrifugation, showed improvement in BBB integrity following hypoxia-ischemia in neonatal brains; the effect was mainly via the transfer of Annexin A1 inside MSCs extracellular vesicles (Gussenhoven et al. 2019). Factors that are transferred within stem cell-derived exosomes for promoting angiogenesis and function of endothelial cells are shown in Fig. 10.5.

10.4.2 Secondary Beneficial Effects of Exosomes

10.4.2.1 Anti-inflammatory and Immune-Related Outcomes

Injuries to the brain (or CNS in general) are associated with a generalized immunosuppression that is associated with poor outcomes in affected patients and favors infections due to defects in lymphocyte activation (Prass et al. 2003; Meisel et al. 2005). In specific, stroke-induced immune suppression is associated with poor prognosis and reduced neurological outcomes (Meisel and Meisel 2011). Strategies to combat this generalized immunocompromised environment are of importance to favorable post-stroke recovery and reduced occurrence of medical complications (Chamorro et al. 2012).

In this regard, exosomes from BM-MSCs were found to reverse this immunosuppression via maintaining normal cellular composition of peripheral circulation (B, T, and NK cells) and attenuating the activation of immune cells (CD69 expressing

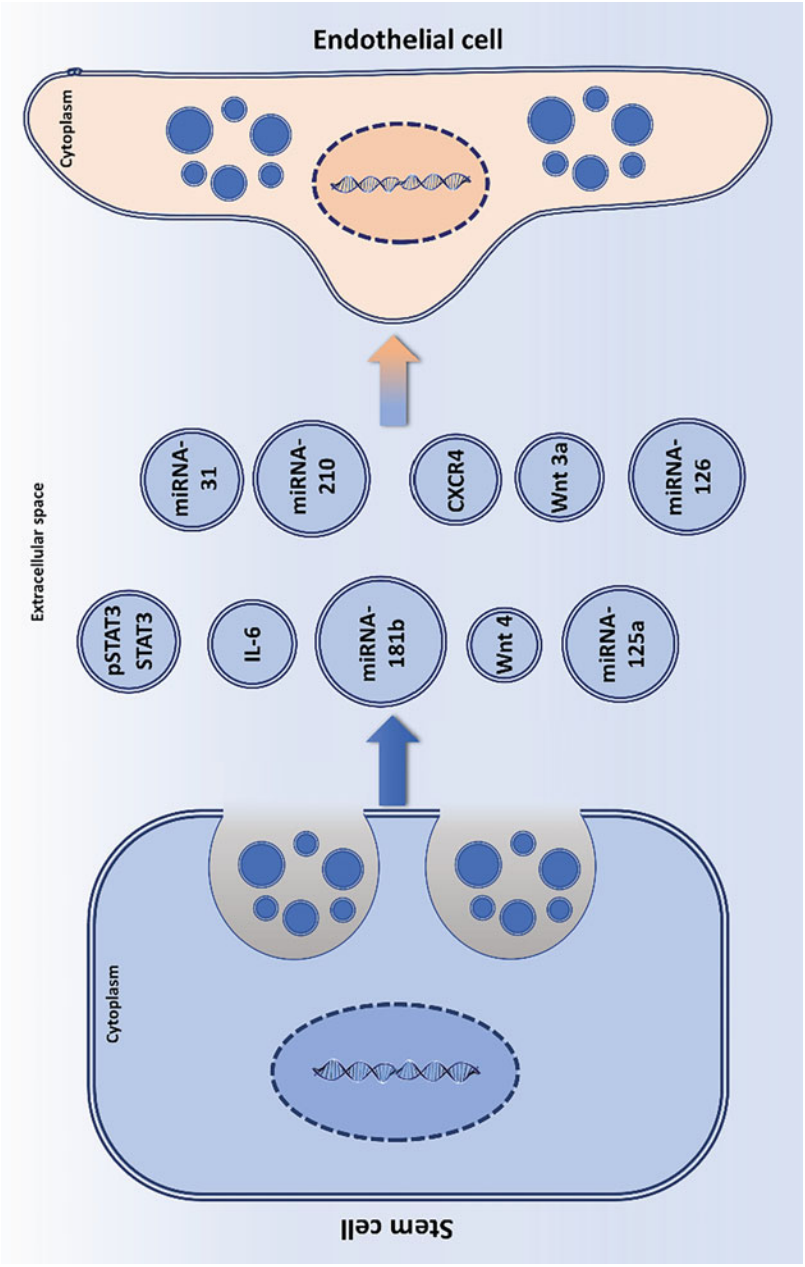

Fig. 10.5 Effects of MSCs produced exosomes on endothelial cells which participate in stimulating angiogenesis following stroke. (Adapted from Bian et al. 2019)

$CD4^+$ and $CD8^+$ T cells) (Doeppner et al. 2015). In contrast, BM-MSCs exosomes did not alter the local recruitment of leukocytes (neutrophils, monocytes, T and B lymphocytes, dendritic cells, and macrophages) into the infarct area of the brain. It is interesting and still not known how exosomes administration in ischemic stroke could show these different effects, where locally it seems not to interfere with the inflammatory processes, however systemically it corrects the post-stroke immunosuppression.

Glia cell's production of immune-regulatory and anti-inflammatory mediators is also affected by the administration of exosomes and exosomes play important roles in the glia-neural communication (Pascual et al. 2020). A recent evidence was obtained when exosomes isolated from primed MSCs (by incubation with interferon gamma, IFNγ) had a strong anti-inflammatory effect on brain microglia cells. This was achieved via the transfer of miR-467f and miR-466q which inhibited p38 MAPK signaling pathway and led to inhibiting microglia activation and the expression of pro-inflammatory cytokines as TNF, IL-1B, IL-6, and nitric oxide synthase (NOS)-2 (Giunti et al. 2021). In another study on spinal cord injury (SCI), activation of neurotoxic A1 astrocytes was reduced following the administration of MSCs exosomes, this was associated with decreased levels of TNFα, IL-1α, and IL-1β and increased expression of myelin basic protein (MBP), synaptophysin (Syn) and neuronal nuclei (NeuN) all indicating reduced inflammation and improved regeneration at the site of SCI (Wang et al. 2018a).

In general, the mechanisms of immune suppression conveyed by MSCs exosomes are, in part, through the packaged immune mediators, such as transforming growth factor-β (TGF-β), interleukin (IL)-6, IL-10, nitric oxide (NO), prostaglandin E2 (PGE-2), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO) and human leukocyte antigen G (HLA-G) (the immune-modulatory mechanisms of MSCs exosomes are summarized elsewhere (Zhang et al. 2014; Burrello et al. 2016; Xie et al. 2020)). As an example, exosomal transfer of miR-138-5p to *in vitro* cultured astrocytes reduced their expression of the inflammatory factors IL-6, IL-1β, and TNF-α (Deng et al. 2019).

10.5 Exosomal Cargo, Importance for Recovery Following Ischemic Stroke

10.5.1 Exosomes Intrinsic Factors

As mentioned above, exosomes possess many intrinsic factors, mainly miRNAs together with mRNAs, lncRNAs, circRNAs, proteins, and others, which can effectively contribute to their noticed healing effects in the studied models for ischemic stroke. These assorted factors incorporated into exosomes will largely depend on the type of cells that produced exosomes and their state/condition during exosome biogenesis.

10.5.2 Modified/Synthetic Exosomes as Vehicles for Drug Delivery

Due to the important biological properties of exosomes, they can be a top candidate as drug vehicles for efficient delivery of extrinsic factors (i.e., drugs, nucleic acids, or other therapeutic agents) (Ha et al. 2016). This is because of their ultra-small size which can pass intact barriers (including BBB) thus they can easily reach the damaged area without being trapped elsewhere, and the fact that exosomes are also low immunogenic, non-toxic and easily biodegradable. This is compared to cell-based therapy where systemically administered cells are trapped in other organs, such as the lungs, spleen, or liver, in addition, these cells start differentiating into other cells thus the number of administered cells is drastically reduced in case of most stem cell-based therapies. Some reports added that exogenous exosomes can be detected in the injured tissues for up to 4 weeks following a single administration. Therefore, exosomes represent an excellent natural candidate for drug delivery purposes.

In this regard, recent studies have focused on overexpressing certain beneficial factors in stem cells (as mRNAs, miRNAs, etc.) or engineering exosomes and loading them with beneficial factors. Exosomes' modifications can be an additional beneficial option which can increase the accuracy of targeted drug therapy and will significantly promote poststroke therapeutic and functional recovery.

Exosomes obtained from MSCs overexpressing miR-133b were found to increase neurite remodeling and synaptogenesis in the ischemic boundary zone and led to considerable improvement of motor functions up to 28 days post stroke (Xin et al. 2017b). In another study by the same group, overexpression of miR-17–92 cluster (such as miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a) in MSCs led to their enrichment in derived exosomes and, compared to normal exosomes from non-transfected MSCs, their use resulted in superior improvement in local neurogenesis and overall functional recovery of mice following ischemic stroke (Xin et al. 2017a). These exosomes containing miR-17–92 cluster targeted the phosphatase and tensin homolog (PTEN) and its downstream targets; including mechanistic target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK-3β), led to improved axonal outgrowth, neurite remodeling, and oligodendrogenesis in the ischemic boundary zone. Similarly, overexpression of miR-126 in adipose-derived MSCs exosomes was related to improved neurogenesis and angiogenesis and reduced glia cell activation and inflammation *in vitro* and in the ischemic areas of the brain *in vivo* (Geng et al. 2019).

Other approaches include the engineering of exosomes and loading them with selected therapeutic molecules. One approach produced exosomes that express transferrin (to facilitate passage through BBB) and loaded it with enkephalin (which confers neuroprotection and neuronal regeneration), these exosomes led to reduced neuronal cell death *in vitro* and promoted functional recovery via increasing neurons numbers and neurological score following ischemic stroke *in vivo* (Liu et al. 2019). Another approach engineered exosomes with rabies virus glycoprotein (to ensure selective delivery to the nervous system) and used to deliver the circular RNA SCMH1 (circSCMH1) to the ischemic brain and the outcome was improved

functional recovery via enhancing neural plasticity, reducing glia activation and reducing immune cells infiltration (Yang et al. 2020). This targeted neural delivery of circSCMH1-containing exosomes was found to be efficient in both rodent and non-human primate models of acute ischemic stroke (Yang et al. 2020).

To enhance the delivery efficiency of exosomes and to increase their passage through the BBB, the surface of exosomes was modified by conjugation to the c (RGDyK) peptide and was found to be effective for their targeted delivery to the ischemic brain areas (Tian et al. 2018). These engineered c(RGDyK)-conjugated exosomes were then loaded with curcumin and their administration led to strong suppression of neuronal cell death and immune responses in the injured brain to demonstrate that surface-modified exosomes can still be loaded with drugs and are still efficient in targeting ischemic brain areas (Tian et al. 2018).

Exosomes were also loaded with drugs that are intended to treat ischemic stroke. An example is edavarone which reduces cerebral infarction following ischemia and has a promising neuroprotective effect but has some limitations as short half-life and low bioavailability. In order to improve its bioavailability and enhance its neuroprotective effects, edaravone was loaded into exosomes derived from macrophages (Li et al. 2020a). Exosomes assisted in increasing the half-life of edavarone and effectively delivered it to the ischemic areas leading to reduced neuronal cell death and microglia activation highlighting their clinical utility.

Therefore, exosomes not only contribute to the transfer of their important intrinsic cargo but also can be loaded with selected molecules and drugs that lead to improved therapeutic outcomes in ischemic stroke models. Future research will reveal the clinical utility of these approaches in human patients suffering from ischemic stroke.

10.6 Exosome Source, Is There a Difference Between Cell Sources?

Since the discovery of mesenchymal stem/stromal cells, the interest in their regenerative potential has been greatly increased. Their therapeutic potential has been, mainly, attributed to paracrine effects rather than direct involvement of MSCs. As mentioned above MSCs-exosomes (and EVs) confer a similar, if not superior in some reports, beneficial effects in healing ischemic stroke and reducing the associated neural damage and loss of function. In these reports, un-modified exosomes were isolated from several cell sources and were used as therapeutic tools, such as bone marrow MSCs, adipose-derived MSCs, peripheral blood MSCs, umbilical cord MSCs, neural progenitor cells derived from embryonic stem cells and, even, macrophages. In addition, engineered exosomes from several sources were also used and proved effective as well. Additional sources for exosomes isolation and therapeutic use in ischemic stroke will be discovered in the future as well. The use of exosomes in ischemic stroke (or any other disease) is also complexed by the differences reported by different research groups in the isolation, characterization, and preparation processes of exosomes for therapeutic uses.

One study compared the effects of exosomes isolated from neural stem/progenitor cells (NSCs) compared to mesenchymal stem/progenitor cells (MSCs); both originating from the same pluripotent stem cell line, and found that NSCs-derived exosomes are superior to MSCs-derived exosomes in a murine model of stroke (Webb et al. 2018). However, the latter study highlighted that this cannot be representative to all MSCs-derived exosomes and more comparative studies are still needed (Giebel and Hermann 2019). Therefore, in the current lack of studies comparing effects of exosomes from different sources and the lack of standard protocols for exosomes isolation, characterization, and preparation for therapy, it still remains to be discovered in the future which exosome source is superior and for which indication it should be prescribed, for achieving the best recovery following ischemic stroke.

10.7 Exosomes in Clinical Trials of Ischemic Stroke

So far, only 4 studies that targeted the use of exosomes were registered in ClinicalTrials.gov website (using the keywords "stroke" and "exosomes", last accessed on 5th of June 2021). One clinical trial is currently recruiting acute ischemic stroke patients to test the therapeutic effects of exosomes extracted from allogenic MSCs (ClinicalTrials.gov Identifier: NCT03384433). The remaining three trials will analyze exosomes and their content as biomarkers in ischemic stroke patients (ClinicalTrials.gov Identifier: NCT04184076, NCT04266639, and NCT03255408). Also, similar to exosomes, few number of clinical trials have investigated cell-based therapy (mainly autologous/allogeneic BM-MSCs, ADSCs, or UC-MSCs) for the treatment of ischemic stroke patients (for more information on stem cells use in clinical studies, please check Chen et al. 2014; Singh et al. 2020; Li et al. 2020b).

In the near future, and with more studies and advancements in exosome research as a therapeutic tool for ischemic stroke, the availability of more data will encourage additional clinical studies to evaluate exosomes' beneficial effects as a powerful diagnostic, prognostic, and therapeutic tool.

10.8 Limitations of Exosome Therapy for Stroke

Exosomes' therapeutic use for stroke and other diseases in general is still in its early stages of development since cell source, dose, route, and time window for exosome administration are all factors affecting the outcomes of their therapeutic success. Additional limitations include the need for standardized techniques for both the large-scale production and isolation of high-purity exosomes at lower costs to make exosome therapy feasible and affordable for all those who need it. Also, when exosomes are used as vehicles for drug delivery, the accuracy of targeting the diseased cells and not healthy cells is another limitation that needs careful investigation as well.

In addition, it is becoming clearer that exosomes contribution to tissue homeostasis (i.e., maintenance and repair) is a complex process which cannot be easily solved with few studies, because, (1) almost all cells in the body can secrete exosomes, (2) intercellular exosome exchange is bidirectional and could be multidirectional as well, and (3) only one cell type is usually studied *in vitro*, whereas *in vivo* the scenery is more complex. Therefore, up until we master the control of exosomes production, what cargos can be sorted inside exosomes and their release mechanisms, it is still far from safe to recommend the widespread therapeutic use of exosomes in ischemic stroke or other life-threatening diseases.

10.9 Conclusions

Our knowledge on exosomes' biogenesis and molecular control of their production is still evolving. Exosomes' importance as diagnostic, prognostic, and/or therapeutic tools for ischemic stroke is growing and their use as drug carriers is also under investigation. There are various bioactive molecules (miRNAs, lncRNAs, circRNAs, etc.) that were identified inside exosomes and can be used as effective biomarkers for IS. More research is required to narrow down these markers to few and combine them into one single diagnostic and prognostic platform. In addition, using exosomes for therapy proved to be effective in animal models of stroke due to their pleotropic beneficial effects; in particular, exosomes derived from MSCs were found to confer neural protection, reduce post-ischemic apoptosis, enhance neurogenesis, stimulate angiogenesis, protect the blood-brain barrier, modulate inflammatory responses, and overall improve the functional recovery following ischemic stroke. Furthermore, exosomes (natural, modified or synthetic) were also recently used as drug delivery vehicles.

In the near future and with more research to improve our understanding of exosomes' use in therapy and more focus on solving the limitations which face exosome therapy; such as the production scale and costs, exosome therapy will revolutionize modern therapeutic approaches for ischemic stroke and many other diseases.

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Role of Nanomedicine in Treating Ischemic Stroke

11

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Abstract

Stroke is a medical condition that occurs due to uninterrupted blood supply to the brain and when the blood supply to part of your brain is interrupted or reduced, preventing brain tissue from getting oxygen and nutrients. It is considered as the second most common cause of death after coronary artery disease. The damage to brain areas poses a *socioeconomic burden and makes the patient disabled. There is no proper cure for stroke; however, prevention or symptomatic treatment may improve the patient's condition, and* no treatment strategies focus on the regeneration of the neurons. Stem cells can be a promising option *in regenerating the neurons in stroke*. Mesenchymal stem cells, neural stem cells (NSCs), embryonic stem cells, and human-induced pluripotent stem cells derived-NSCs can be used as a treatment option in stroke. Nanotechnology has an important role in the monitoring of stem cell therapy. Nanomedicines have been used for theranostic purposes to prevent further activation of ischemia; their surface functionalization properties make them amenable in theranostics and targeting treatment approaches.

Superparamagnetic iron oxide nanoparticles (SPIONs), MPIOs, ION, inorganic quantum dots (qds), aggregation-induced emission (AIE) dots, nanodiamonds, micron-sized superparamagnetic iron oxide particles (mpios), superparamagnetic iron oxide nanoparticles (spions), fluorescent-magnetite-nanocluster (FMNC), and semiconducting polymer nanoparticles (spns) are some of the most explored nanostructures in stroke. There are several limitations and advantages of this therapy that need to be understood which requires

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thorough research. We further have discussed the mechanisms of nanoparticles action in stroke and their current challenges and prospects of nanomedicine-based stroke treatment including their clinical and preclinical perspective.

Keywords

Nanomedicine · Ischemic stroke · Stem cells · Iron oxide nanoparticles

11.1 Introduction

The root of stroke was found since the time of Hippocrates which was coined as apoplexy at those times. It was recognized by the symptoms of unconsciousness due to the result of a blockage in carotid arteries (Donkor 2018). The World Health Organization in the year 1970 defined stroke as "rapidly developed clinical signs of focal (or global) disturbances in the cerebral function, which lasts for more than 24 hours or leading to death, with the only cause of vascular origin" (Sacco et al. 2013). Due to advancement in imaging techniques over the period, the latest definition of transient ischemic attack (TIA) is as follows "a short-term episode of neurological dysfunction began by focal brain or retinal ischemia, with clinical symptoms lasting less than one hour, and without indication of acute infarction" (Easton et al. 2009). In the year 2013, ischemic stroke is the 2nd leading cause of death after ischemic heart disease. Data shows that there are 25.7 million survivors in the year 2013, and a maximum of them were having ischemic stroke (Feigin et al. 2017). Furthermore, in 2016, it gains the same second position leading to the death of 5.5 million people. On the scale of disability-adjusted life years (DALYs), it is in 2nd position having the highest incidences in the East Asian region followed by the eastern European region and lowest incidences in the central Latin American region (Gorelick 2019).

Leaky vasculature and BBB are hurdles in stroke therapy as even nanomaterials can revert to the systemic circulation which may precipitate side effects. So, here, challenge comes about how to exploit the leaky BBB; furthermore, some nanoparticles themselves may even exacerbate BBB leakage. Impairment of BBB membrane integrity and associated brain edema was also observed in some studies (Sarmah et al. 2017). Recent pathological advances show that ischemic stroke affects neurons in the gray matter as well as white matter leading to neuronal loss. Furthermore, restoring the cerebral blood flow also leads to cascades of secondary events which will lead to inflammation and can also lead to compromised veracity of the BBB. The ischemia-induced ischemic cascade comprises a sequence of biochemical actions, such as ion imbalance, energy failure, excitotoxicity, immune response, oxidative stress, initiation of inflammation, activation of the complement system, cell death (apoptosis or necrosis), etc. Ultimately, multimodal irreversible neuronal death continues either by apoptosis, necrosis, or autophagy (Xing et al. 2012). Even if stroke patients survive after the stroke attack, it causes neurological deficits in stroke survivors which leads to socioeconomic burden (Nucci et al. 2020).

Treatment approaches for ischemic brain injury aim to achieve reperfusion, neuroprotection, and neuro recovery (Benedek et al. 2019). However, reperfusion exacerbates ROS production, amplification of inflammation, and immune response which causes impairment in BBB integrity and eventually leads to brain edema. Traditional treatment strategies mainly involve antithrombotic drugs and neuroprotective agents. The treatment strategies applied immediately are intravenous (IV) alteplase, antihypertensive, and ultimately fibrinolytic. After the first stroke incidence, the further incidence is avoided by decreasing risk factors through decreasing risk factors like hypertension, hyperglycemia, and atherosclerosis (Powers et al. 2018). But to date, no treatment strategies focus on the regeneration of the neurons, or the available strategies are in the developmental phase (Negoro et al. 2019). However, stem cell therapeutics have been suggested for use in combination with approved thrombolytic or thrombectomy approaches (Nucci et al. 2020). To date, the regenerative aspect of the treatment is widely unaddressed, stem cells can be a major outbreak, and they can be a promising option (Kwak et al. 2018). Therapeutic angiogenesis cell replacement, neuroprotection, endogenous neurogenesis, and modulation on inflammation and immune response are some of the regenerative approaches representing a promising tool to improve the prognosis of cerebral ischemia (Benedek et al. 2019; Hao et al. 2014).

Stem cells that can be used for stroke treatment are mesenchymal stem cells, neural stem cells (NSCs), embryonic stem cells, and human-induced pluripotent stem cell-derived NSCs (Marei et al. 2018). Clinical trials named MASTERS and TREASURE, ACTIsSIMA, Pisces, I-Act, and CoBIS2 are some of the promising ongoing clinical trials (Krause et al. 2019). Some of the major issues of stem cell therapies are a selection of route of administration, assessment of its efficacy, effective administration in the infarcted area, and slow pace due to stringent regulatory requirements (Zhang and Yao 2017; Liska et al. 2017). Apart from this word developmental issues, cost, time, and difficulties in recruitment and retention of patients have also been noted (Krause et al. 2019).

Advancement in nanotechnology leads to the development of nanostructures and devices which can be applied in various fields ranging from diagnostics to therapeutics. Considering the diagnostic arm of the treatment, it can be used as an analytical and imaging tool as well as a theranostic agent too. Moreover, it is used for targeted drug delivery and can also be used in tissue engineering and regenerative medicine (Patra et al. 2018). From the perspective of the usage of stem cell medicine in ischemic stroke, nanoparticles can be used for tracking and monitoring of stem cells that ultimately leads to the determination of its efficacy (Zheng et al. 2018). One of the widely used nanoparticles for tracking and monitoring stem cell therapy is superparamagnetic iron oxide nanoparticles (SPIONs) (Duan et al. 2016; Lin et al. 2017). A meta-analysis was carried out by articles published from January 2000 to October 2014 showing 24 articles that were then analyzed based on stem cell type, nanoparticle characteristics, and effectiveness in animal models. Various reports concluded that nanotechnology has an important role in the monitoring of stem cell therapy (Nucci et al. 2015). Another approach for the enhancement of stem cell therapy is biomaterials. Biomaterials provide a highly compatible microenvironment

for neural tissue regeneration and can be used as a carrier for stem cells as well as other neuroprotective agents (Tang et al. 2015). Biomaterials used for the treatment of stroke are proteins, glycosaminoglycans, and synthetic protein (Love et al. 2019). A study carried out on mice by inducing stroke through middle cerebral artery occlusion model (MCAO) and treating mice by injecting high cluster density nanoparticles of vascular endothelial growth factor (VEGF) within hyaluronic acid hydrogel at lesion site shows the development of vascular bed and ultimately the axons (Nih et al. 2018). A meta-analysis carried out on a biomaterial-based approach for ischemic stroke till 4 February 2019 concluded that they have a positive effect on histological and neurological aspects in preclinical studies (Bolan et al. 2019).

11.2 Stem Cells for Regenerative Therapy of Stroke

A regenerative approach for the treatment of stroke appears to be promising, and it appears to provide functional recovery through mechanisms like neurogenesis, angiogenesis, neuroprotection, and immunomodulation (Kwak et al. 2018). Adult mesenchymal stem cells, adult embryonic stem cells, and neural stem cells show excellent distinction toward neuronal phenotypes (Marei et al. 2018). Out of the total 6116 clinical trials of stem cell therapy, 44 are of stroke, i.e., 0.73%. Out of those 44, 27.3% are active trials, 38.6% are completed, 25% are withdrawn, and 9.1% are terminated (Krause et al. 2019). The clinical trials have been increased involving assessing the efficacy of cell therapy in functional and structural renewal after stroke. However, no adequate data in the literature showing the best route, best cell type to be used, and the best nanoparticle to analyze these stem cells in vivo (Nucci et al. 2015). Embryonic stem cells (ESCs), neural stem cells (NSCs), inducible pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs) have been used in animal studies that demonstrated the beneficial effects of stem cells on stroke (Hao et al. 2014). In contrast, mesenchymal stem cells (MSCs) are known as a promising approach for acute ischemic stroke which is cell-based therapy (Lin et al. 2017) (Fig. 11.1).

11.2.1 Mesenchymal Stem Cells (MSc)

Mesenchymal stem cells (MSCs) differentiate into neural-like cells (NCs) (Leder et al. 2015). MSCs are the most explored cells in clinical and preclinical settings and can be resultant from various sources like the placenta, muscle skin, bone marrow, adipose tissue, and dental pulp (Marei et al. 2018). As it has been extensively explored in clinical settings, it is hence considered as safe, and it also shows regenerative capacity (Han et al. 2020). Bone marrow-derived mesenchymal stem cells (BM-MSc) are one of the major stem cell types utilized in ongoing clinical trials (Kwak et al. 2018). One of the clinical trials conducted named ACTIsSIMA sponsored by SanBio uses SB623 cells (BM-MSc) for treating stroke. The route used was intracranial, and after 12 months of follow-up of recruited patients, it shows

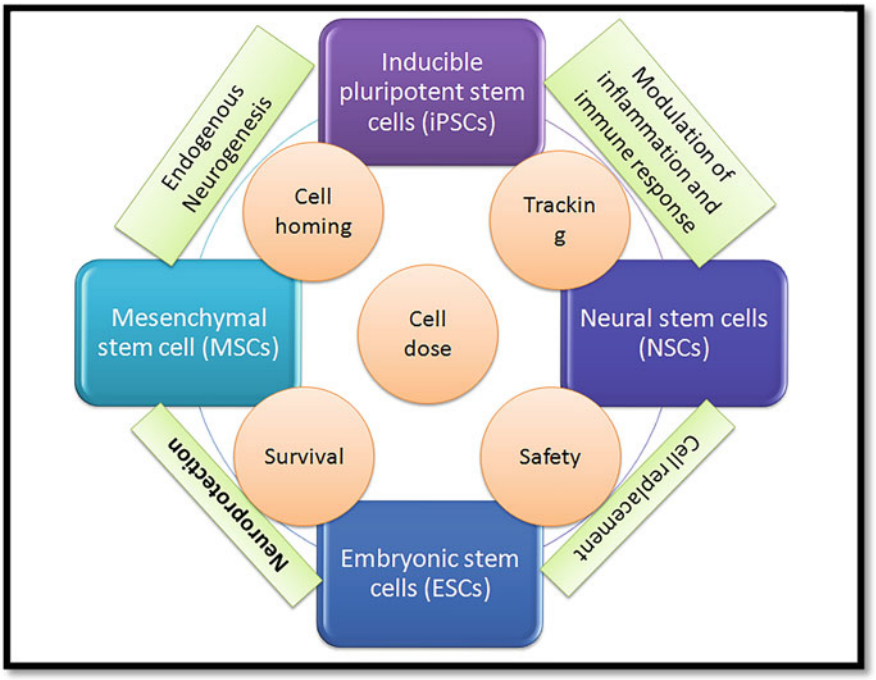

Fig. 11.1 Beneficial effects and limitations of various stem cells employed in animal studies

improvement on various measurement scales of stroke in phase1/2a trials. Phase 2 was started in 2016 and is estimated to be finished by 2019 (Krause et al. 2019).

11.2.2 Neural Stem Cells (NSc)

Natural neuronal regeneration in mammals occurs in some particular area of the brain; they are called "niches" and include sub-ventricular zones of lateral ventricles, the sub-granular zone of the hippocampal dentate gyrus, and the external germinal layer of the cerebellum. NSc can be extracted easily from the neural tissue and can be easily grown with the help of serum-free media having basic fibroblast growth factor and epidermal growth factor as a supplement. Moving onto the efficacy part of the human-derived neural stem cells (hNSc), it shows improvement in behavioral aspects of the MCAO stroke model especially in rats (Churcher et al. 2017). After its effectiveness was proved in preclinical studies, one of the hNSc reached the clinical phase in a trial named PISCES. The cells named CTX-DP are given to 11 patients through stereotaxic ipsilateral putamen injection at the dose of 2, 5, 10, or 20 million cells, and after the completion of 2 years of study, its safety is established without any treatment-emergent adverse event. Results of the PISCES 2 phase 2a trial which started in 2014 are available on ReNeuron's website but not published as

a peer-reviewed publication. PISCES 3 phase 2b started in 2018 as per clinicaltrials.gov, and it is estimated to be finished by November 2022.

11.2.3 Embryonic Stem Cells (ESc)

Neural precursors were developed after human embryonic stem cells (hESc) with the help of a small molecule smad inhibition protocol. Further, they are developed to functional neurons in an in vitro setting, and after its transplantation in vivo, it attenuates regeneration and sensory recovery (Drury-Stewart et al. 2013). As hESc is pluripotent and is self-renewable, they have a potential for the production of a large number of neuronal cells, but when directly transplanted, the proliferative nature becomes a threat for tumor formation (Machado-Pereira et al. 2018). Another threat with the use of embryonic stem cells is their immunogenic rejection (Kwak et al. 2018).

11.2.4 Induced Pluripotent Stem Cells (iPSc)

They were first developed by Dr. Yamanaka from mouse fibroblasts and reprogrammed for pluripotency same that of embryonic stem cells (Takahashi and Yamanaka 2006). Differentiation of the cells will lead to the replacement of damaged neurons, but another aspect that is related to the use of iPSc is its tumor generation potential same as embryonic stem cells. One add-on advantage with the iPSc is that it loses immunogenicity after the process of differentiation. To date, no clinical trial for stroke using iPSc has been reported.

11.3 Challenges in Stem Cell Therapies for Ischemic Stroke

In the arena of stroke therapeutics, the neurorestorative aspect is the recent one, and to execute it, the main tool in our hand right now is stem cells. As they are a novel approach, it has many bottlenecks and shortcomings attached to them. Looking at the broader picture: the main issues with the stem cells remain limited available bases of stem cells, the time window for the therapy, inherited limitation of adult stem cell potential, and aftereffects of transplanting like tumors (Bang et al. 2016). Apart from determining the safety and efficacy of the desired stem cell, the translation of the route of administration from animals to humans is a major challenge (Krause et al. 2019). Furthermore, the problem with transplanted cells is their survivability in vivo. Major factors affecting cell survivability are anoikis, oxidative stress-mediated apoptosis, and immune rejection (Sart et al. 2014). Another bottleneck is tracking the efficacy of the cells when they are transplanted in the brain. To assess the bioavailability and distribution of the transplanted cells, live imaging and tracking systems are needed to be developed with the use of tracking agents which are safe for the human body (Tang et al. 2015). Ultimately, this helps to know the

dynamics after the transplantation of the cells and also the detailed mechanism of action. Techniques used for in vivo imaging are magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET), and optical imaging through fluorescence and bioluminescence {Zhang 2017 #30}. As mentioned, the main issues with stem cell therapy are its survivability and its monitoring after the transplantation. Nanoparticles could be a better option to track the in vivo activity through various imaging techniques like MRI (Nucci et al. 2015). Furthermore, to enhance the survivability of the stem cell, biomaterials approach seems to be more promising (Xu et al. 2019).

11.4 Tracking Stem Cells

Stem cells can be tracked easily by labeling them either directly or indirectly. Direct labeling includes nanoparticles, fluorescent dyes, or radionuclide, while indirect labeling includes genetic modification. Direct labeling of the cells is easy, cheap, and with less risk of mutagenesis with easy translation to human subjects, whereas indirect labeling is more expensive, has risk of mutagenesis, and requires molecular biology expertise. The disadvantages of direct labeling are that they can't give information about in vivo proliferation and false signal generation if it penetrates host tissues (Jasmin de Souza et al. 2017).

An inseparable part without which tracking of cells is not possible is imaging systems. Different imaging systems work based on different tracking agents, for example, MRI can be well used with the SPION, and it can also be used for guiding injection to a particular place for stroke treatment. Gadopentate dimeglumine is the first MRI contrast agent approved by USFDA. This compound proves to be clinically safe as more than 99% of its dose is excreted out through the renal pathway. Furthermore, when stem cells are labeled with gadopentetate dimeglumine, it decreases the renal excretion rate and may be retained in the body for a longer period (Bulte and Kraitchman 2004). Hence, there are needs for such compounds which can be used for proper tracking of the cells and could be relatively safe. Different molecular imaging techniques and their particular labels are mentioned in the below table (Table 11.1).

Table 11.1 Imaging techniques and labels used

Molecular imaging techniques	Cell labels
PET	Molecules containing radionuclides ^{11}C, ^{13}N, ^{18}F, ^{76}Br
SPECT	^{99m}Tc, ^{123}I labeled compounds
MRI	SPIONs and gadolinium complexes
Fluorescence imaging	Fluorescent proteins, fluorescent dyes, and antibodies conjugated with fluorescent dyes
Bioluminescence imaging	Luciferase/luciferin

As advancement in the material sciences is very rapid, it influences many other various fields, and diagnosis is no exception. Nanoparticles are part of the advancing nanomaterials field and have immense application in various pharmaceutical sectors. As stem cells are recent technology for treating ischemic stroke, new and advanced tracking systems are also required with safe labeling agents and nanoparticle-based labels being one of the approaches. Nanoparticles like SPIONs, micron-sized superparamagnetic iron oxide particles, and fluorescent magnetite nanoclusters are used for tracking stem cells in stroke models with the help of MRI, and they show a promising response in the long-term tracking and checking out the efficacy of the stem cells. Furthermore, they have been utilized with almost every type of stem cells, and due to their noninvasive nature, they can be of great help to stem cell treatment (Zhang and Yao 2017). Therefore, such imaging modalities must use safe tracer agents and should be noninvasive. Also, to control the optimum therapy for stroke in relationships of the route of administration, dosage, and timing, the imaging requires image resolution and high sensitivity collective with continuing monitoring (Zhang and Yao 2017).

11.5 Role of Nanoparticles in Stroke Therapy

Nanomaterials have unique utility potential in stroke due to their specific properties such as long circulation time, crossing biological barriers, enhanced targeting properties, etc. (Blanco et al. 2015; Weissleder et al. 2005). However, this uniqueness depends upon shape, size (from 5 to 1000 nm), composition, conductivity, monomers molar ratio, surface charge, hydrophilicity, the rigidity of nanotools used, and their release in the different basic and acidic environment inside cells (Bonnard et al. 2019). Nanomedicines help in improving pharmacokinetic behavior so that the maximum drug reaches the site of action requiring less amount of drug and thus reduces the side effects. Brain targeting in stroke should involve drug delivery to the damaged brain parenchyma or thrombus site specific to the neurons or glial cells. Also, nanomedicines offer options for some novel therapies including gene therapy that helps recovery even in the chronic phase of stroke. After coming to the systemic circulation, nanomedicines also protect the drug against enzymatic degradation, and the relatively large size of nanomedicines averts the drug from being rapidly removed by the kidney.

Nanomaterials help in stroke therapy by the following mechanisms: (1) enhancing thrombolysis, (2) limiting bleeding in hemorrhagic stroke, (3) decreasing neuronal death, and (4) diagnosis prognosis of stroke (Fig. 11.2).

Other pathomechanisms targeted by nanomaterials help in stroke therapy which include nanomedicines regulating ion disparity and excitotoxicity, nanomedicines transporting oxygen to the ischemic brain, nanomedicines falling oxidative stress, nanomedicines managing inflammation and immune response, nanomedicines regulating inflammatory cells, nanomedicines reducing apoptosis, nanomedicines preventing pro-inflammatory mediators, nanomedicines regulating multiple

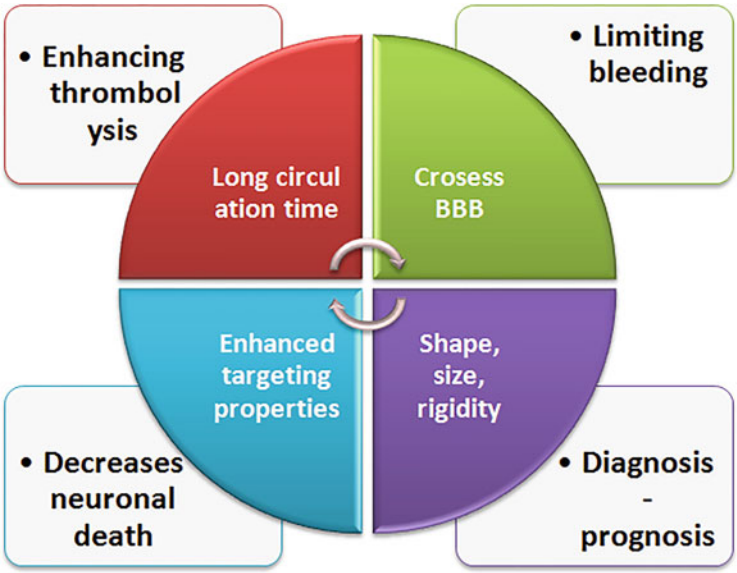

Fig. 11.2 Specific properties and mechanism of action of nanomaterials use in stroke therapy

abnormalities, nanomedicines promoting tissue compensation, and nanomedicines for pretreatment with TNF-α.

11.5.1 Nanomedicines Transporting Oxygen to the Ischemic Brain

Adequate O_2 delivery to the ischemic penumbra is a possible technique for patients who are not appropriate for thrombolysis or revealed microcirculation damage. Liposomal Hb (LHb) has been used to overcome the limits of hemoglobin (Hb) that has a hypertension response and short half-life. In addition to lengthening the circulation time of Hb in vivo, it also delivers enough oxygen to the penumbra.

11.5.2 Nanomedicines Regulating Excitotoxicity and Ion Imbalance

Glyburide is an inhibitor of the SUR1-TRPM4 complex reducing edema and infarct volume, but it has the limitation of BBB crossing. Therefore, thrombin-responsive size-shrinkable brain directing NPs, poly (L-lactide-co-glycolide) nanoparticles (PLGA-NPs), betulinic acid loaded with glyburide NPs was synthesized to target ATP sensitive nonselective cation channel (SUR1-TRPM4) which causes depolarization upon ATP depletion (Dong & Feng, 2007).

ZL006 is another drug used to ameliorate excitotoxicity by blocking the nNOS-PSD-95 interaction selectively as postsynaptic density protein-95 (PSD-95) contributes to the initiation of neuronal nitric oxide synthase (nNOS); but, it also

has the drawback of accumulation in the ischemic brain. Therefore, dual targeting of ZL006 was done by conjugating liposomes with stroke-homing peptide (SHp) and T7 peptide, where T7 peptide helped liposomal transport to BBB through TfR-mediated transcytosis and SHp-mediated endocytosis of liposomes via glutamate receptors and gets preferentially accumulated in damaged/ischemic territory (Dong et al. 2020).

11.5.3 Nanomedicines Reducing Oxidative Stress

Oxidative stress is responsible for several pathologies related to neuronal diseases. Phyto-derived bioactive compounds play a role in the prevention and treatment of neuronal disorders. However, their low concentration is not able to reach the CNS, and thereby it limits stability, bioavailability, and dissolution at target sites. Thus, nanosizing enhances the permeability into the CNS with maximized stability and efficiency (Ganesan et al. 2015). Several antioxidants such as baicalin, luteolin, curcumin, resveratrol, tanshinone, and puerarin have been employed to construct nanoparticles overcoming their limitation which not only scavenge excessive ROS nevertheless but also decrease downstream apoptosis and inflammatory responses.

A recent study has shown the antioxidant properties of selenium nanoparticles compared to their bulk form (Kondaparthi et al. 2021). Nano-antioxidants such as carbon nanotubes, metal and metal oxide nanoparticles, and various types of polymer-loaded antioxidant nanoparticles are accorded as effective therapeutic and prophylactic agents. Liposomes are also used to deliver antioxidants due to their amphiphilic nature. Antioxidant functionalized nanoparticles including silver nanoparticles (AgNPs), gold nanoparticles (AuNPs), copper oxide nanoparticles (Cu_2ONPs), iron nanoparticles (INPs), zinc oxide (ZnONPs), selenium (SeNPs), and nickel oxide nanoparticles (NiONPs) have been used for delivering various biological extract (Kumar et al. 2020).

11.5.4 Nanomedicine Reducing Apoptosis

Bio-macromolecules are more researched anti-apoptotic drugs which have been delivered using ligand-modified NPs and exosomes. Reducing apoptosis weakens further immune response and inflammation as it's an intermediate event in stroke. A recent study has also observed the anti-apoptotic potential of α-bisabolol loaded solid lipid nanoparticles against Aβ-induced neurotoxicity in neuro-2a cells (Sathya et al. 2020). Ce^{3+} ions from cerium oxide nanoparticles (nanoceria) have shown Ce^{3+}/Ce^{4+} redox-dependent anti-apoptotic effect (Celardo et al. 2011). Cerium oxide (CeO2), silver nanoparticles (AgNPs), zinc oxide nanoparticles (ZnO NPs), and yttrium oxide (Y2O3) nanoparticles have shown anti-apoptotic effects (Hosseini et al. 2013; Sanaeimehr et al. 2018; Yuan et al. 2018). Bioactive proteins human VEGF and human angiopoietin 1 co-encapsulated in human serum albumin nanoparticles demonstrated a significant highly proliferative and anti-apoptotic

effect (Khan et al. 2011). The anti-apoptotic activities of SeNPs were demonstrated by decreased Bax and caspase-3 expression (Alkhudhayri et al. 2020).

11.5.5 Nanomedicines Regulating Immune Response and Inflammation

Inflammation is an essential immune reaction that empowers survival and maintains tissue homeostasis. Nanomedicines are loaded with a therapeutic anti-inflammatory agent for targeting inflammation. They work either through the recognition of molecules overexpressed onto the surface of activated macrophages or endothelial cells or through biomimicry or even through improved vasculature permeability. Steroid-based nanomedicines, non-steroidal anti-inflammatory drugs (NSAIDs) nanomedicines, nano-encapsulated anti-inflammatory mediators, anti-inflammatory peptides nanomedicines, nanomedicines for gene therapy, biomimetic nanoparticles, and nanoparticles for immune system modulation are some examples of new innovative nanomedicine systems for preclinical therapies against inflammatory disorders (Brusini et al. 2020). NPs modulate immune function leading to immunosuppression or immunostimulation that may bring benefits or danger (Jiao et al. 2014). Different physical and chemical properties of metal-based nanoparticles induce different cellular responses, leading to different immune responses (Luo et al. 2015).

In addition to stopping vital mechanisms in the inflammatory reaction nano medicines also show exhibits anti-inflammatory effects.

11.5.6 Nanomedicines Inhibiting Pro-inflammatory Mediators

Pro-inflammatory mediators involve *pro-inflammatory cytokines* including TNF-α, IL-1β, IL-8, as well as CD86 protein, pro-inflammatory macrophages, etc. Downregulation of pro-inflammatory mediators is required to resolve the inflammation. The levels of pro-inflammatory mediators which are target components of the immune response are constantly high in chronic inflammation. Prednisolone phosphate encapsulated in PEGylated liposomes was shown to downregulate the *pro-inflammatory mediator.* Liposomal formulations have been shown to decrease the pro-inflammatory mediators in an in vivo study (Prasad et al. 2015).

Nanogold (AuNGs) has been shown to reduce the level of pro-inflammatory cytokines TNF-α and IL-1β (Khan and Khan 2018).

Gene silencing by small interfering RNA (siRNA) regulates the specific protein functions but in vivo limits their therapeutic application.

11.5.7 Nanomedicines Regulating Cells Involved in Inflammation

Inflammation is an immune response that allows the tissue to heal following the resolution of the infection or injury. It can be resolved by immune modulation using targets ranging from antigen-presenting cells to activated T cells, macrophages, and B cells. Macrophage repolarization strategy for inflammatory disease focuses on repolarization as a strategy for anti-inflammatory therapy, macrophage polarization spectrum, and macrophage-targeted delivery systems (Talekar et al. 2015).

Nanomaterials can be used in several other approaches of therapeutics such as tissue engineering, regenerative medicine, biomaterials, transplantation, and stem cell biology for treating inflammation. Polymers conjugated with drugs or tissue targeting molecules to proteins encapsulated within a polymer shell, nanomedicine is used for cell-based therapeutics (Samarasinghe et al. 2012).

This includes PEGylated Ce-NPs to polarize M1 to M2 phenotype microglia. PEGylated Ce-NPs were typically triggered M1-phenotype microglia and have pro-inflammatory action; whereas, otherwise stimulated M2-phenotype microglia may employ anti-inflammatory effects.

11.5.8 *Nanomedicines Promoting Tissue Repair* Add more literature here in terms of stroke

Biomaterials and nanostructures in nanotechnology have been involved in regenerative medicine. The biggest challenge in CNS diseases involved nerve regeneration. Nanotopography or orientation of the bio-composite nanofibrous scaffolds made from synthetic and natural polymeric blends greatly influences the nerve cell morphology and outgrowth. Many materials, synthetic and natural, have been established for engineering nerve conduits; however, selection of the appropriate material is essential to reproduce the specific characteristic of the native nerve. Neurotubes have been designed for peripheral nerve repair or reconstruction (Perán et al. 2013).

Growth factors and targeted delivery of microRNAs (miRNAs) that stay intricated in the development of neuronal transformation have been used to mediate neurogenesis (Fig. 11.3).

11.6 Carbon Nanotubes (CNT)

These are a new class of nanomaterials that have been shown their applicability in CNS. Because of their outstanding physicochemical properties, carbon nanotubes CNTs have been employed as a theranostic tool with functionalization of carbon nanotubes, stimuli-responsiveness, and carbon nanotube-based biosensors (Komane et al. 2016).

The plausible utility of these nanomaterials has been suggested regarding regenerative matrices of neuronal tissues and for the CNS delivery of therapeutics (Nunes

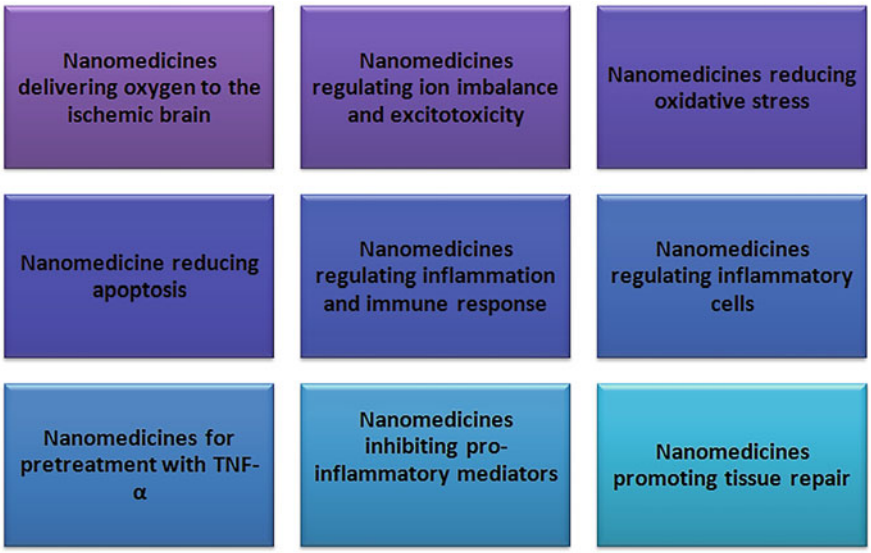

Fig. 11.3 Nanomedicines targeting pathomechanisms involved in ischemic stroke

et al. 2012). N-cadherin is an essential protein for cell survival, and adhesion has continued high in nanotube-exposed rats (Mattson et al. 2000).

11.6.1 Single-Walled Carbon Nanotubes (SWNTs)

It has been seen that nanotubes reduce stroke damage. Pretreatment of rats with single-walled carbon nanotubes has shown protective effects in neurons and enhanced the recovery after stroke injury (Higgins et al. 2011). They also showed decreased levels of neuronal apoptotic markers in rats pretreated with amine-modified single-walled nanotubes. Besides, decreased post-stroke glial and inflammatory responses show the potential of nanotubes in limiting cell death and inflammation (Lee et al. 2011).

11.6.2 Multi-Walled Carbon Nanotubes (MWCNT)

MWCNTs are used as transporters for small interfering RNA (siRNA) mediated gene silencing as they are biocompatible and effective. Studies have shown a 200% surge in neurite length upon growth on multi-walled carbon nanotubes (MWCNT) and a 300% rise in coated MWCNT in comparison to uncoated nanotubes (Mattson et al. 2000).

11.7 Liposomes

Due to the neuroprotective properties of xenon, it has been employed as a brain delivery therapeutic for stroke therapy in xenon-encapsulated liposome form using ultrasound guidance (Sarmah et al. 2017). Different types of nanocarriers were used to deliver plasminogen activators (PAs) mostly targeting fibrin or platelets of thrombi. For example, PAs were encapsulated into platelet targeted liposomes showing similar efficacy to free PA with a substantial reduction of bleeding side effects (Zhang et al. 2018). However, surface-functionalized PAs have shown a potentiated fibrinolytic effect than vehicle incorporated (Juenet et al. 2018). Nanoparticles targeting involves physical, biomimetic, adsorptive-mediated transcytosis, and ligand-mediated active targeting. Nanocarrier-based brain targeting involves decorating nanomaterials surface with peptides or aptamers with BBB receptor-ligand delivery system, coupling antibodies, BBB receptor ligands, and transporter ligands united micelle delivery (Fig. 11.4).

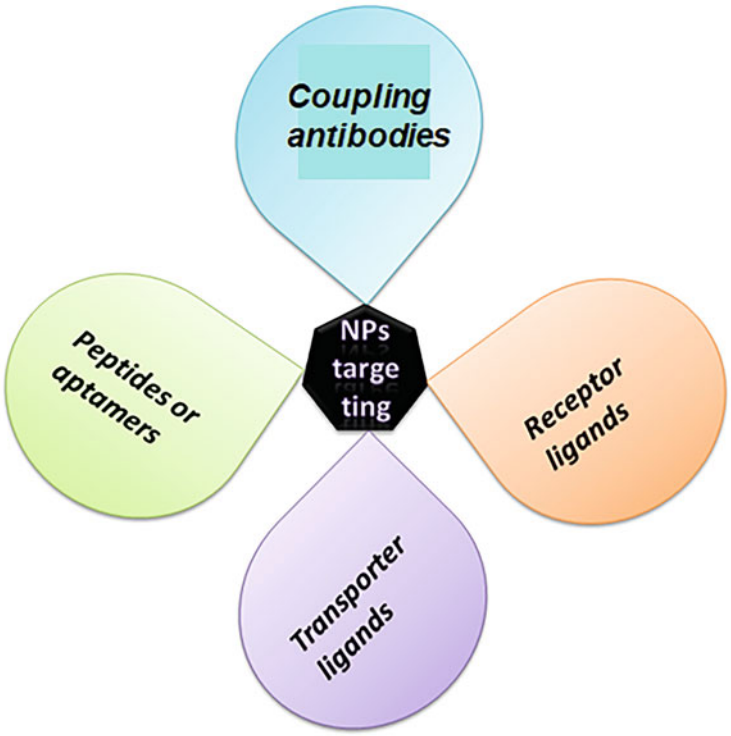

Fig. 11.4 Nanocarrier-based brain targeting approaches

- Nanospheres and Polymeric Nanoparticles

This has also been used increasingly as an agent for stroke therapy. PNP materials are (1) synthetic polymers and (2) natural polymers. Synthetic PNP polymers include poly(lactic acid), poly(glycolic acid), poly(D, L-lactide-co-glycolide acid), polycaprolactone, and its polyethylene glycol derivatives: poly(lactic acid)-polyethylene glycol (PEG), poly(D, L-lactide-co-glycolide acid)-PEG, and polycaprolactone-PEG, while natural macromolecular polymers include chitosan, gelatin, starch, etc. (Chen and Gao 2017). Polymeric nanoparticles (PNPs) have been used to limit bleeding in hemorrhagic stroke utilizing poly(lactic-co-glycolic acid)-poly-L-lysine block copolymer core with polyethylene glycol arms terminated with Arg-Gly-Asp (RGD) functionalities (Bertram et al. 2009). Biodegradable PNPs are considered as potential drug delivery carriers for administering protective drugs such as thyroid hormones, retinoic acid, osteopontin, PEGylated epidermal growth factor, and erythropoietin and thus reduce neuronal death as compared to a free drug. One study showed high mobility group box-1 (HGMB1) siRNA delivery using PAMAM-Arg, containing polyamidoamine (PAMAM) dendrimer amide attached with basic L-arginine residues (e-PAM-R). e-PAM-R/siRNA complex has shown a significant decrease in HMGB1 levels, neuronal cell death, and reduced infarction sizes in the rat MCAO model (Kim et al. 2010).

11.8 Hydrogels

Hydrogels modulate the crosslinking density and thus match the mechanical characteristics of the normal brain. It can be transplanted directly into the stroke cavity and offers structural support to the damaged tissue. It bypasses the BBB by guiding the injection via noninvasive imaging and promotes local regeneration by promoting infiltration of parenchyma cells around (Lemmens and Steinberg 2013). Hydrogels have been seen as an emerging treatment option for brain repair after stroke. It is injected as a liquid and solidify in situ to form a gelatinous solid formed by reducing the mobility of water-swollen polymers via the introduction of physical and/or covalent bonds between polymer chains, creating a crosslinked network. It provides structural support to the surrounding tissue to minimize secondary cell death and manage the inflammatory response (Nih et al. 2016). Microporous annealing particle (MAP) hydrogel injection in the stoke cavity has shown reduced gliosis and inflammation and promoted NPC migration to the lesion (Nih et al. 2017b). Cook, D. J., et al. have studied the effects of hydrogel-delivered brain-derived neurotrophic factor shown to promote tissue repair and recovery after stroke. Nih, L. R., et al. engineered hyaluronic acid (HA)-based hydrogel for selective control of human neural stem cell survival and differentiation after transplantation in the stroke brain (Nih et al. 2017a). Hyaluronic acid particle hydrogels also have shown decrease in cerebral atrophy and promote pro-reparative astrocyte/axonal infiltration in the core after ischemic stroke (Sideris et al. 2019).

11.9 Cell-Derived Nanovesicles

Mesenchymal stem cells (MSC)-derived exosomes and extracellular nanovesicles (NV) have multifaceted therapeutic benefits such as induction of angiogenesis, anti-apoptosis, and anti-inflammation and therefore may be used for the treatment of ischemic stroke. Cell-derived nanovesicles are advantageous over artificial carriers because of their targeting abilities and better biosafety. A brain directing peptide (T7 peptide) related erythrocyte membrane nanovesicles laden through Mn_3O_4 nanoparticles (Mn_3O_4@nanoerythrocyte-T7, MNET), the composite has shown free radical scavenging properties, long circulation time, ability to cross BBB, and change hypoxia environments in the ischemic brain (Shi et al. 2019). L-arginine and γ-Fe_2O_3 magnetic nanoparticles (PAMNs) loaded Platelet membrane-derived nanovesicles were used for both therapeutic and diagnostic purposes. Upon application of an external magnetic field, PAMNs delivered L-arginine which encouraged vasodilation besides disrupted the thrombosis for reestablishing the blood flow (Li et al. 2020).

However, its clinical application needs large-scale production that requires developing new technologies. Currently, cell-derived NV has been used to deliver anti-inflammatory agents; however, interesting studies to deliver lipid mediators have been conducted which requires further detailed investigation (Dinarello 2010).

- Other Therapeutics Using Nanotechnology

Liquid squaline conjugated adenosine nano-assemblies have shown improvement in neuro deficits in ischemic animals (Gaudin et al. 2014).

Nanoparticles in Stem Cell Tracking

Inorganic quantum dots (qds), nanodiamonds, aggregation-induced emission (AIE) dots, micron-sized superparamagnetic iron oxide particles (mpios), superparamagnetic iron oxide nanoparticles (spions), fluorescent-magnetite-nanocluster (FMNC), semiconducting polymer nanoparticles (spns), etc. are mostly used rationally designed nanoparticles explored for constant or real-time tracking of stem cells employing biocompatible contrast agents (Ni et al. 2020).

11.9.1 Quantum Dots

Unique properties of quantum dots are emerging as optimal tools in long-term MSC optical imaging applications. Quantum dots (QDs) as fluorescent probes are fluorescent semiconductor nanoparticles with unique optical (e.g., narrow band emission spectra and broad excitation spectra) and chemical features (e.g., resistant to chemical and metabolic degradation) making them useful as fluorescent tags for long-term in vitro and in vivo cell imaging applications (Kundrotas et al. 2019). Quantum dot-based fluorescent probes are considered relatively cheap preclinical assessment of cell migration in vivo. NIR emitting quantum dots provides an excellent means for

preclinical, long-term study of cell migration (Gavins and Smith 2015). Thus, these optical contrast agents are employed as drug delivery and bioimaging agents for labeling molecules, cells, and tissues; however, they show toxicity toward cultured cells (Sarmah et al. 2017). Near-infrared silver sulfide (ag-2 s) quantum dots were used in the tracking of transplanted human mesenchymal stem cells in living mice (Chen et al. 2014). Carboxylated quantum dots were investigated in human bone marrow MSCs at different cell growing densities, and the results show them as promising in fundamental stem cell biology as well as in cellular therapy (Kundrotas et al. 2019). Intracellular delivery of quantum dots also aids in live-cell labeling and organelle tracking due to their ability to target the nucleus and mitochondria were studied (Derfus et al. 2004).

11.9.2 Nanodiamonds

Nanodiamonds have properties like biocompatibility, highly stable photoluminescence, and a surface structure that can be easily modified to facilitate bioconjugation. Nanodiamonds have been proposed for use in the development of therapeutic agents for diagnostic probes, targeted delivery vehicles, gene therapy, tissue scaffolds, antiviral and antibacterial treatments, labeling of cells for tracking, and protein purification (Blaber et al. 2013).

11.9.3 Iron Oxide Particles

Since the 1980s, iron oxide particles have been in use for cellular and molecular magnetic resonance imaging. In previous research, micron-size particles were used. After a long disappearance because of their stability and improved construction, utility of MPIOs has been rediscovered. Ferucarbotran, a clinically available ION, is used for tracking implanted cells visualized under MRI (Naqvi et al. 2009).

11.9.3.1 Micron-Sized Superparamagnetic Iron Oxide Particles (MPIOs)

MPIOs because of their very high iron content are used as a cell-tracking agent. MPIOs enable the recognition of single cells, single elements in single cells, and the ability to label cells directly, in the body. USPIOs and SPIOs are more commonly used iron oxide particles. MPIOs have been used as a tagging agent for monitoring bone marrow-derived mesenchymal stromal cell (BMSC) migration in the brain using magnetic resonance imaging (MRI) in a rat model of stroke. But, the detection of iron particles may not be a suggested appropriate strategy for the recognition of BMSCs in the brain as the actual origin of cells containing iron oxide particles remains unclear. Proof of concept has been proposed for microRNA-based manipulation involving multifunctional, silica-based micron-sized iron oxide-containing particles (sMPIO) that conglomerate MRI tracking, imaging fluorescence, and on-the-spot directing of exact microRNAs on a particle surface aimed at therapeutic management by RNA intervention. These theranostic particles provide a promising tool for cell transplantation (Leder et al. 2015).

11.9.3.2 Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

Superparamagnetic iron oxide nanoparticles (SPIONs) are used for diagnoses in stroke because of their apparent safety for humans and their unique properties. Still, its long-standing in vivo effects have continued to be examined. They can be professionally used in labeling and for noninvasively tracking cells by magnetic resonance imaging later transplantation (Jasmin de Souza et al. 2017). To observe and survival and migration of stem cells, magnetic resonance imaging (MRI) is an attractive and clinically translatable tool. The common intracellular delivery SPIONs via poly-L-lysine (PLL) need a high SPION concentration and a long incubation time for suitable cell labeling that affects the function and viability of stem cells. To overcome such limitations, researchers have developed cationic polymersomes for cellular MRI in acute ischemic stroke. These superparamagnetic iron oxide-loaded cationic polymersomes for cellular MR imaging had comparable biological safety, labeling efficiency, a marginal advantage on post-transplantation cell endurance, a substantially shorter labeling period, and relatively low SPION concentration. On the other hand, MRI overvalued the actual dimension of the cell grafts. Thus, SPION-loaded cationic polymersomes have been suggested to be used as a substitute for rapid, safe, and efficient labeling of stem cells for cellular MRI (Duan et al. 2016). Researchers also developed a biocompatible nanocomplex (ASP-SPIONs) constructed using cationic amylose, utilizing presenting spermine and the image tagged, ultrasmall superparamagnetic iron oxide nanoparticles (SPIONs), to label MSCs. Apart from small SPIONs concentration, with short labelling time ASP-SPIONs, the biocompatible complex were synthesized and thus is highly translatable for clinical application. The capacity, efficiency, cytotoxicity of the nanocomplex in transferring SPIONs into green fluorescence protein-modified MSCs, presentation of in vivo MRI following of the transplanted cells, etc. also need to be investigated.

11.9.3.3 Fluorescent Magnetite Nanocluster (FMNC)

Fluorescent magnetite nanocluster has been developed to label and track MSC due to its high MRI sensitivity. These novel nanoclusters have been designed for their utility in the labeling and tracking of mesenchymal stem cells (MSC). FMNC was constructed by embedding individual magnetite nanoparticles (NPs) into a polystyrene scaffold coated with two layers of silica and a sandwiched layer of rhodamine. FMNC has shown high cell-labeling efficiency with no adverse effects on MSCs. FMNC-labeled MSCs migrated and accumulated in the ischemic region after FMNC labeled MSC transplantation in a mouse middle cerebral artery occlusion model (Wang et al. 2011).

11.9.4 Black Phosphorus

Black phosphorus (BP), namely, phosphorene, has application in the field of photoacoustic imaging, photothermal therapy, and fluorescence imaging (Xie et al. 2018) (Fig. 11.5).

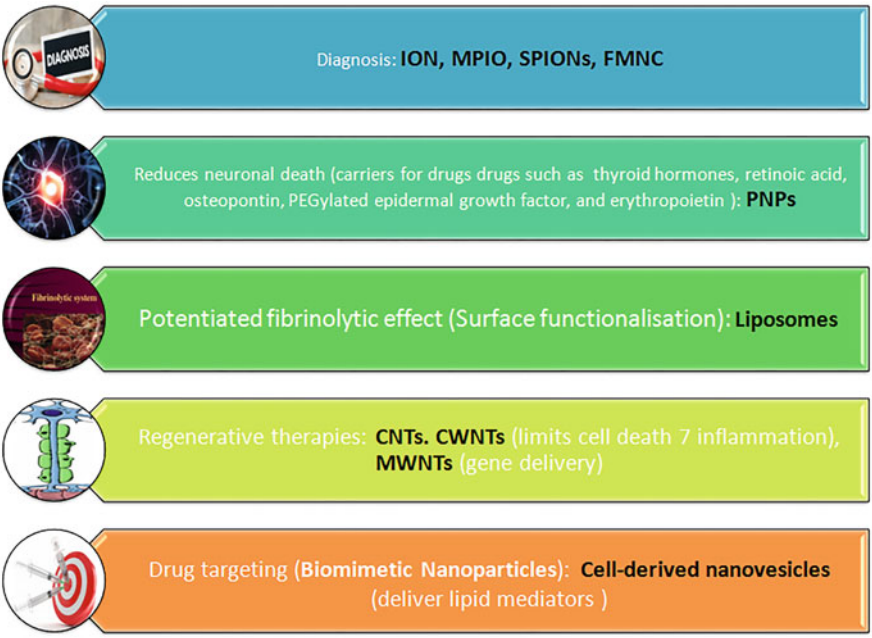

Fig. 11.5 Nanomaterials used in stroke for specific purposes

11.10 Prospects and Challenges of Nanomedicine-Based Stroke Treatment

Controlling drug delivery and the development of novel delivery vehicles are vital reasons to improve stroke therapy. Nanoparticles are utilized to increase drug solubility and tissue targeting (Dong et al. 2020). Larger-size nanomedicines may be opsonized through plasma proteins and then afterward phagocytosed through the reticuloendothelial system (RES), and thus nontargeted accumulation can occur in the spleen and liver. However, PEGylation may perhaps support evade phagocytosis, accomplish a longer circulation period in the blood, and diminish the indefinite distribution of nanomedicines (Naqvi et al. 2020).

Nanomedicines are important molecules in stroke therapy because of their ability to transiently decrease the BBB paracellular tightness, cross the BBB, actively transport by transcytosis, and act as antioxidants. As nanomaterials are foreign materials, hence, their toxicological, pharmacological effects, interactions with the human brain, and biodistribution studies should be conducted in detail after systemic administration. A very limited number of animals and clinical studies exploring the toxicity of nanomaterials in the brain are available. Surface functionalization is also playing an important role in deciding neurotoxicity in the case of nanomaterials and increases the risk of encouraging toxic substances in the brain. Carbon nanotubes

have shown toxicity and liposomes, and hydrogel has shown limited negative effects in animal studies involving stroke. Also, recruitment of randomized clinical trials (RCTs) for acute stroke is difficult, and a sufficient amount of sample is limited. In vivo short half-life, low solubility, and deprived BBB permeability of most neuroprotective agents might subsidize the failure of many clinical trials. Moreover, intracranial or intrathecal injection, unsustainable drug concentration limits their utility, and systemic administration studies is a further difficult in most cases. Various nanoformulations such as dendrimers, nanocrystals, polymers, micelles, SLNPs, liposomes, etc. nanoparticles have been used; however, it is rather difficult to say which nanoparticulate system has more potential and safe in stroke therapy. Brain targeting has an inherent limitation of target proteins also significantly expressed in peripheral organs.

11.11 Summary

Stem cell therapy in together clinical and preclinical trials is known as a hopeful possible therapeutic approach in the treatment of cerebral ischemia. Currently proposed stem cell treatments consist of the replacement of lost neurons, the release of growth factors, and the secretion of extracellular vesicles in recipient cells and promote endogenous repair processes. It requires elucidating the precise mechanisms underlying stem cell therapy and the migration, homing, differentiation, and distribution of transplanted stem cells in vivo utilizing various imaging techniques. Time window, cell dose cell homing, survival, tracking, and safety should be addressed for stem cells in stroke management.

However, thorough research is required to measure intravascular stem cell therapy as a potential adjunct to mechanical thrombectomy or thrombolysis in ischemic stroke.

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Insights into Therapeutic Targets in Stroke

12

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Abstract

A stroke is also called a cerebrovascular accident which damages the brain from interruption of blood supply. Vast research has been developed to date to develop accurate treatment and alternatives to only one FDA-approved therapy, t-PA. Also, recent techniques such as stem cells for regeneration and imaging techniques for early diagnosis of stroke have been employed. However, the research has gained limited success in achieving desired results. It's because of the multifaceted pathophysiology of stroke and limitations of novel or existing therapies along with the inappropriate preclinical design and drug discovery of experiments. Nanotechnology applications in stroke research have shown promising effects in preclinical stages. They are advantageous due to their surface modification properties and to overcome the limitations of existing therapies. They are used for a theranostic purpose as a drug delivery carrier, contrast agent for imaging, gene delivery, coating agent, or targeting agent. However, this technique requires an in-depth understanding of the use and selection of carriers and their pharmacokinetics, toxicity, sensitivity, selectivity, capacity to be imaged with commercially available equipment, etc. Understanding the pathophysiology of stroke is very crucial for the selection of targets, choice of therapeutic agent, selection of nanoformulations, etc. as it involves multiple pathways. Moreover, a combination of drugs or selection of drugs targeting multiple targets is also important and has to take into consideration. More research is required to make wise use of these techniques to translate to the clinical trials and make them successful in stroke therapy. So, in this book

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chapter, we have covered the pathophysiology-based therapeutic approaches with a particular focus on ischemic penumbra. Molecules released and can be targeted in salvageable ischemic penumbra include apoptotic markers, free radicals, ion channels, glutaminergic signaling, activated protein C, GABA A receptors, antioxidant levels, reperfusion, etc.

Nanomaterials used as a contrast agent in the diagnosis of stroke include metal nanoparticles, quantum dots, polymeric nanoparticles, dendrimers, etc. with their characteristics and examples. Nanoparticles used as drug carriers in stroke therapy include carbon nanotubes, metal nanoparticles, polymeric nanoparticles, liposomes, quantum dots, dendrimers, graphene, black phosphorous, hydrogels, etc. Further, their limitations and approaches are discussed. Nanomaterials target receptors such as EPOR, TfR, CACR4, LDL, and lactoferrin receptor. Future perspectives in stroke therapy are also covered which have to be taken into consideration while selecting the diagnostic and therapeutic strategy for stroke.

Keywords

Therapeutic targets · Pathophysiology of stroke · Ischemic penumbra · Nanomaterials

12.1 Introduction

A stroke happens when a blood vessel in the brain breaks and bleeds, or the blood supply to any part of the brain is interrupted otherwise reduced, preventing the brain tissue from getting oxygen and nutrients hereby decreasing the quantities of glucose and oxygen reaching this organ resulting in brain cell death in minutes. Decreased levels of oxygen and glucose cause a drop in ATP manufacture through oxidative phosphorylation in the mitochondria that subsequently decreases entire ATP levels in 2 min subsequent to the insult. Thereby, ATP-dependent ionic pumps don't work properly, therefore, impairs maintenance of electrochemical concentration gradients and thus causes the impairment in the main function of the neuron to conduct electrical impulses. This sudden loss of membrane potential in the ischemic core is termed anoxic depolarization (AD) characterized by the opening of voltage-gated Ca^{2+} channels and neuronal death (Bonventre 1988).

Ca^{2+} triggers the release of neurotransmitters (NTs) majorly excitatory NT glutamate into the synaptic cleft. Glutamate then reacts with its ionotropic and metabotropic receptor and initiates action potentials in the postsynaptic nerve cell. Sodium-dependent glutamate transporters in pre-and postsynaptic membranes maintain the proper glutamate concentration thereby involved directly in regulating cellular signaling. Glutamate reaches its neurotoxic levels upon hitch of the action of the glutamate transporters which averts the reuptake of glutamate into the cell.

In addition, the core spreads electrical waves well-known as peri-infarct depolarizations (PID) in the ischemic penumbra, leading to fast de- and repolarizations of these neurons which is correlated with the extent of infarction.

Thus, the objective of dropping the occurrence of PIDs in ischemic penumbra helps as an impending target for therapeutic interference thereby decreasing the number of "at-risk" neurons.

There are two main types of stroke: (1) ischemia (infarction) and (2) hemorrhage. Among them, nearly 87% of all strokes are ischemic. Stroke is a highly heterogeneous neurological disorder involving multiple pathomechanisms. Such processes inducing cell death include ionic imbalance, glutamate-mediated excitotoxicity, peri-infarct depolarization, apoptosis, oxidative stress, etc. The t-PA is the solitary FDA-approved treatment for ischemic stroke that has a fewer therapeutic window of around 4.5 h even after intravenous administration. This strategy dissolves the clots but doesn't prevent further reperfusion injury which is related to a 6% danger of intracranial hemorrhage. Moreover, exogenously administered t-PA can cross the blood-brain barrier and may also improve neurodegeneration due to its capability to work together with the NR1 subunit of glutamate receptors particularly, *N*-methyl-D-aspartate (NMDA), thus encouraging calcium influx that can afterward extent neurotoxic levels. Glutamate further increases cytosolic Ca^{2+} levels by acting on NMDA receptors. High cytosolic Ca^{2+} levels activate cellular cascades which leads to cell death.

Therefore, drugs with extended therapeutic windows are required which also help in suitable diagnosis and thus taking necessary therapeutic actions. Advances in radiographic imaging are one of the opportunities to treat patients. Fortunately, one of the approaches that have been tried combines both diagnosis and treatment called "theranostics" and allows for a broader application to clinicians in the treatment of ischemic stroke victims. Prototypic brain damage involves the inner core where ATP levels are exhausted and neural cells experience necrotic cell death and the surrounding ischemic penumbra which is functionally impaired. So, high therapeutic window drugs provide the opportunity to salvage the ischemic penumbra as ATP levels are not completely exhausted in this area, but this area is disposed to undergo apoptosis. Therefore, the size of this target must be considered before the origination of treatment, for instance, the amount of the core associates to clinical aftermaths (Stankowski and Gupta 2011).

Nanomaterials help in the delivery of various therapeutic agents which are not able to cross BBB because of their large size which is not allowed by smaller endothelial gaps. Stroke is an inflammatory response that causes endothelial activation and neutrophil infiltration. Novel nanoparticles have been designed (gold, polymeric, or protein nanoparticles) which utilize neutrophil infiltration to transport nanoparticles across the blood vessel. Receptors are expressed on the brain surface such as immunoglobulins receptors, transferrin receptors, insulin receptors, growth factors, and low-density lipoprotein receptors, responsible for transporting large molecules. G-protein-coupled chemokine receptor-4 (CXCR4) and matrix metalloproteinase-2 (MMP-2) are the molecules that are highly expressed in the stroke microenvironment (Dong et al. 2020).

Irreversible injury to the core infarct can be salvaged by quantifying cerebral blood flow (CBF) by use of positron emission tomography (ct) as the irreversibly injured core has a CBF fewer than 12 cc/100 g/min which can no longer be salvaged.

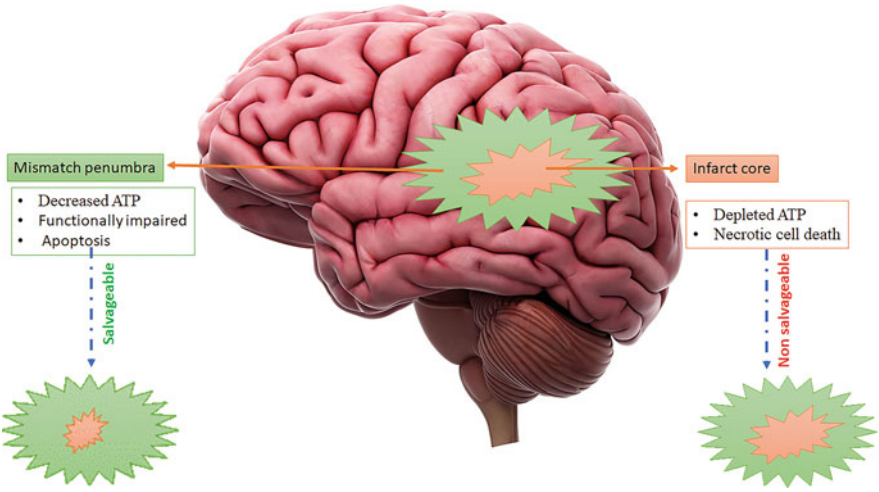

Fig. 12.1 Salvageable ischemic penumbra is the therapeutic target in stroke

The mismatch is the core of the emphasis of clinical research in reperfusion treatment which is bigger penumbra compared to the core. Xenon CT is readily available imaging quantifying CBF in the clinical situation than PET. MRI perfusion and CT cannot precisely define the penumbra as they are qualitative but defines populations with mismatch; therefore, thresholds are being developed.

Understanding molecular processes that are activated in stroke can reduce the bottlenecks in the drug development and discovery process and thus speed up the process with safe and effective medications coming to the clinical trials and their approval for use in public. Understanding the pathomechanisms is also an important aspect in designing a nanotherapeutic based multidrug approach (Fig. 12.1).

12.2 Therapeutic Targets in Stroke

12.2.1 Ischemic Penumbra

A range of molecules has been targeted which are involved in ischemic penumbra including apoptosis (Bcl-2 family members, caspases, and poly(ADP-ribose) polymerase (PARP)), which is activated by signals such as ROS generation, ionic disparity, caspase stimulation, DNA damage, mitochondrial swelling, etc. Therefore, proper diagnosis requires the identification of these events at initial hours in ischemic penumbra, so further cell death can be prevented (Stankowski and Gupta 2011).

12.2.1.1 Apoptosis

Cell death involving necrosis or apoptosis has to be evaluated 1–10 days after the ischemia using the terminal deoxynucleotidyl transferase-mediated dUTP nick end

labeling (TUNEL), 2,3,5-triphenyl tetrazolium chloride (TTC) stain, hematoxylin-eosin (H & E) staining, and caspase-3 staining.

12.2.1.2 Caspases

Caspases are cysteine aspartyl proteases involved in both origination and execution stages of apoptosis that form a proteolytic cascade acting a fundamental part in neuronal death. It confirms that caspase inhibition embraces tremendous neuroprotective potential in apoptosis-related degenerative diseases and stroke. Active caspase-3 existed in the ischemic cortex of the brain, and thus Z-DQMD-FMK, its specific inhibitor, weakened beta-catenin damage, but it did not interrupt phosphorylation of both beta-catenin and glycogen synthase kinase-3beta.

12.2.1.3 Free Radicals

Free radicals are reactive oxygen species (e.g., non-radicals and superoxide (O(2) *−), such as reactive nitrogen species and hydrogen peroxide. Overproduction of ROS and RNS has been shown to harm various cellular constituents, including lipids, DNA, and proteins. Antioxidant therapy along with factors such as dose, blood-brain barrier (BBB) penetrability, and administration time should be taken into consideration. Hydroxy radical scavenging edaravone has shown significant functional improvement and shown equivalent effectiveness with that of its control drug sodium ozagrel (ozagrel).

12.2.2 Ion Channels

Blocking cation channels is one of the approaches used to provide therapeutic benefit in stroke, but they have not so far produced significant clinical advantage. The problems involved regarding research include the use of a stroke preclinical model, which does not adequately compete with the clinical condition and the deficiency of small-molecule blockers of these ion channel families (Tuttolomondo et al. 2009).

12.2.2.1 Na^+ Channels

Blocking Na^+ channels offsets main proceedings in stroke. Lidocaine, BW-1003C87, tetrodotoxin, BW-619C89, and lamotrigine can decline the rate of ATP depletion and lessen glutamate release. Hypothermia has been seen as a therapeutic target upon failure of Na^+ channel blockers in clinical trials. Hypothermia (30 °C) in animal models showed a significant decrease in the extent, size of infarction, and several depolarizations (Stankowski and Gupta 2011).

12.2.2.2 Ca^{2+} Channels

SB 201823-A has been shown the ability to block calcium influx in vitro. The efficacy of isradipine, L-type calcium channel blocker, has also been evaluated in numerous other prototypes of global and focal ischemia: mouse MCAO, gerbil

bilateral carotid artery occlusion (BCAO), and rat rose bengal (Tuttolomondo et al. 2009).

12.2.3 Modifications of Glutamatergic Signaling

Blockage of presynaptic glutamate release as well as the use of NMDA antagonists has been found neuroprotective in preclinical models of stroke. AMPA receptors are resistant to Ca^{2+} under their GluR2 subunit, but the change in this subunit initiates delayed neuronal cell death. In contrast, glutamate acts indirectly on metabotropic glutamate receptors (mGluRs) and initiates various downstream signaling cascades and contributes to excitotoxicity. Therefore, the use of mGluR5 antagonists in decreasing excitotoxicity is suggested as an additional potential target in neurotherapeutics development. The links have been found among excess intracellular Ca^{2+} concentration and the initiation of nerve cell nitric oxide synthase (nNOS) which causes an increase in amounts of intracellular nitric oxide (NO). It further improves the reactive oxygen species (ROS) formation, which can ultimately damage nucleic acids, proteins, and lipids. Furthermore, isoprostanes and 4-hydroxy-2-nonenal (4-HNE) are products of lipid peroxidation which subsequently damage other cellular components. Lubeluzole, a NOS inhibitor, has not shown effectiveness in stroke. However, BN 80933 affecting both lipid peroxidation and nNOS has shown more hopeful results (Stankowski and Gupta 2011).

Excessive glutamate release results in activation of p44/42 mitogen-activated protein (MAP) kinase and tyrosine kinase. MAP kinase-regulated pathways promote polypeptide growth factor-mediated neuronal endurance or decease, dependent upon the cellular perspective in which they are triggered. Glycine agonist site-selective antagonist of the NMDA receptor complex kynurenic acid is used for the inhibition of neuronal cell loss.

Although cyclooxygenase-2 (COX-2) enzymes were found to be elevated in excitotoxicity and inhibitors of COX-2 are beneficial, side effects were observed on their use.

12.2.4 Activated Protein C

Thrombin on the external of the endothelial cells causes physiological activation of protein C involving the thrombomodulin and endothelial protein C receptor (EPCR); 2 membrane receptors, thus, activates anticoagulant activated protein C (APC). Additionally, APC extraordinarily acts straight on cells and exerts change of gene expression profiles, antiapoptotic activity, anti-inflammatory actions, and protection of endothelial barrier function.

12.2.5 GABA Receptors A

The gamma-aminobutyric acid (GABA) receptor stimulation effect is undefined in ischemia brain damage. However, studies have been carried out on the effect of the activity of GABA receptors in inhibiting NMDA receptor-mediated nitric oxide (NO) making via neuronal NO synthase (nNOS) in ischemic brain damage. Studies have shown the neuroprotective effects of muscimol GABA(A) receptor agonist and its mixture with baclofen the GABA(B) receptor agonist which significantly protects neurons against death (Tuttolomondo et al. 2009).

12.2.6 Antioxidant Levels

Prooxidant and antioxidant balance is maintained using antioxidants under normal physiological conditions. Inequality is mentioned as "oxidative stress" which produces destructive effects of ROS on cellular stress. Antioxidant therapeutics have been tried aimed at preventing the detrimental effects of ROS on survival and cellular integrity. However, these antioxidants didn't show any protective effects in humans and can also have other side effects (e.g., bilobalide (EGB 761), which is neuroprotective in transient ischemia, however, increases intracerebral hemorrhage.

12.2.7 Reperfusion

Although rapid reperfusion harbors a variety of risks that can deteriorate the problems, it is of greatest importance to rescue brain tissue (Stankowski and Gupta 2011; Fig. 12.2).

12.3 Nanotechnology in Stroke Diagnosis

Magnetic resonance imaging (MRI) and computed tomography are the most preferred methods for diagnosis of stroke because of the advancement to detect intracranial vessel occlusion, evaluate the ischemic penumbra, etc. (Dong et al. 2020). Other methods include computed axial tomography, positron emission tomography scan, or single-photon emission computed tomography (SPECT) (Kyle and Saha 2014). However, Intravascular photoacoustic (IVPA) and Computed tomography (CT) can accomplish higher spatial resolution compared to MRI. Intravascular photoacoustic (IVPA) is a marginally invasive ultrasound-based tomography technique that used gold nanoparticles as a contrast agent. However, their solubility and stability are improved by PEG functionalization (Sharma et al. 2019).

Identifying the proper cause of stroke is essential to select drugs for thrombectomy. However, this technique requires the use of contrast agents to reveal biological processes that constitute potential diagnostic or therapeutic targets (Dong et al. 2020). Cerium oxide, iron oxide, platinum, or perfluorocarbon nanoparticles to

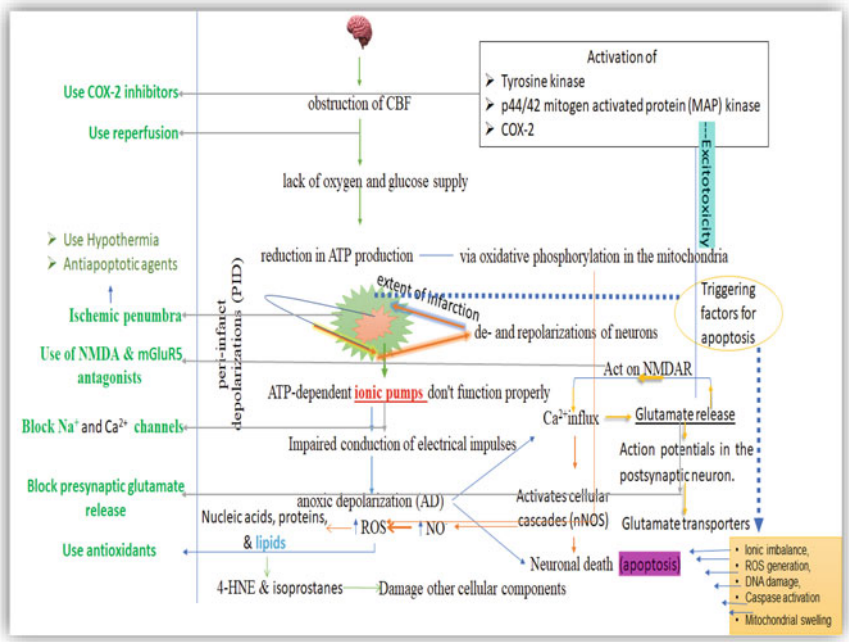

Fig. 12.2 Pathomechanisms and their therapeutic targeting in stroke. (Wu et al. 2020)

quantum dots are commonly used contrast agents in imaging (Kyle and Saha 2014). Thus, nanotechnology has of great use in designing and use of contrast agents in stroke diagnosis. Nanoparticles are used for the diagnosis of numerous biomarkers such as neurotransmitters and ROS in stroke (Dong et al. 2020; Fig. 12.3).

12.3.1 Metal Nanoparticles

Gold, iron oxide, and silver metal nanoparticles are used mainly for imaging applications.

12.3.1.1 Microsized Iron Oxide (MPIO) Particles

Microsized particles of iron oxide (MPIO) are a new family of contrast agents that allowed a large increase in sensitivity and specificity of this MRI. The use of microsized particles instead of ultrasmall superparamagnetic iron oxide particles prevents the passive leakage of particles in the brain parenchyma and increases the payload of contrast material per particle. Noninvasive detection of definite proteins expressed by the cerebrovasculature is done using MPIO coupled to monoclonal antibodies. Monoclonal antibodies are used along with MPIO targeted against adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), which provide etiologic assessment

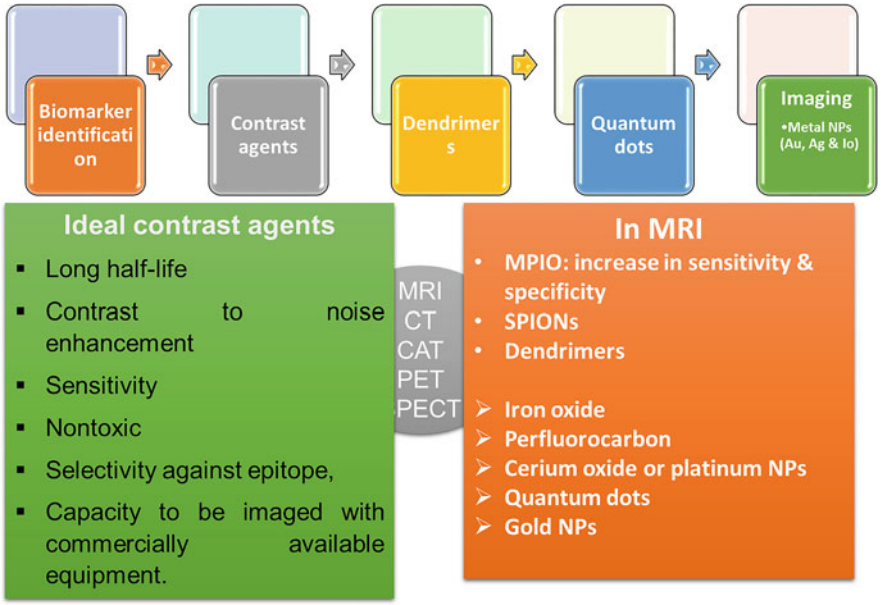

Fig. 12.3 Nanomaterials used as a contrast agent in stroke diagnosis

using the existence of molecular events in stroke. Diagnosis of transient ischemic attack molecular MRI of P-selectin using MPIO is used to detect the endothelial activation and thus distinguish transient ischemic attack from stroke imitators, such as migraine or epilepsy. Therefore, the high sensitivity and specificity offered by MPIO-enhanced molecular MRI make the research focused on translating this method to clinical imaging. However, coating and inner structure make them nonbiodegradable, and hence, the development of biocompatible MPIOs is mandatory (McAteer and Choudhury 2013).

Computed tomography is less sensitive than MRI; still, it is used for molecular imaging using nanoparticles (Bonnard et al. 2019). BBB MR imaging showed the effectiveness of ferumoxtran-10 nanoparticles. This is also used for tracking and targeting human mesenchymal stem cells (Shcharbina et al. 2013).

12.3.1.2 Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

Superparamagnetic iron oxide nanoparticles are available commercially such as VSOP-C184 coated with citrate (VSPIO), ferumoxytol coated with polyglucose sorbitol carboxymethyl ether, and frumoxide coated with dextran (SPIO), which have been used as contrast agents for MRI. SPIONs revealed no toxicity, have long circulating half-life, cross the BBB, identify the pathological or anatomical target, and get cleared by phagocytic RES cells; however, clearance depends upon the size and coating. Thus, SPIONs can be coated with PEG which impedes uptake by RES and delays circulation (Mahmoudi et al. 2011).

12.3.1.3 Perflurocarbon Nanoparticle (PFC)

Perflurocarbon nanoparticles possess most of the ideal properties of imaging agents such as long circulating half-life, sensitivity, nontoxicity, a contrast to noise enhancement, selectivity against epitope, capacity to be imaged with commercially existing apparatus, etc. These are synthesized by replacing hydrogen with fluorine atoms which can further be functionalized for therapeutic action or imaging. PFC can convey a very high paramagnetic cargo, and incorporated fluorine is extraordinarily sensitive to microenvironmental and molecular changes (Cormode et al. 2009).

12.3.2 Quantum Dots

These are semiconductor materials whose optical spectrum can be controlled by varying the size of the core as their absorption and emission spectra are size-dependent. Therefore, they have been used in place of old-style organic fluorophores as simple fluorescent reporters in fluorescent imaging applications, immunoassays, microarrays, and other assay platforms. These are used as contrast agents for bioimaging monitoring drug delivery and as drug delivery mediators to label tissues, biological fragments, and cells. These are very promising fluorescent labels that hold exceptional solidity of optical properties upon pairing to biomolecules. Synthesized quantum dots are much more steady and brighter fluorescent labels that release in the infrared band. Optical admittance to the vasculature and its monitoring is provided noninvasively using quantum dots (Zrazhevskiy et al. 2010).

12.3.3 Polymeric Nanoparticles

Poly-D, L-lactic-co-glycolic (PLGA) formulated polymeric nanoparticle which is Eco-friendly polymer captured with a hydrophilic dye [fluorescein isothiocyanate (FITC)]. Transmission electron microscopy utilizes FITC-encapsulated nanoparticles for highly efficient nanoparticles (Kyle and Saha 2014).

12.3.4 Dendrimers

Dendrimers are designed by monomeric subunit divisions diverging to all borders from a central nucleus, and the structure turns into globular and densely crowded at the margin with increased generation. Many functional groups in dendrimers are accountable for their high reactivity and solubility. They are used as drug-delivery carriers, contrast agents for MRI, drugs for the management of neurodegenerative disorders and prion diseases, novel synthetic vectors aimed at gene therapy, and detoxication agents, antiviral agents, antioxidants, and antibacterial immunostimulant apparatuses (adjuvants). However, no data on their application in stroke is available. Among all nanomaterials, dendrimers are the least toxic and those are characterized for hemolytic toxicity, cytotoxicity, hematologic toxicity, and interaction with protein. Therefore, nontoxic dendrimers have been synthesized

by using a critical nanoscale design parameters strategy (Shcharbina et al. 2013). In MRI, dendrimers are used as coating agents to advance their redistribution in the body. An anti-inflammatory agent N-acetyl cysteine was delivered using PAMAM dendrimer that showed an order of extent advanced antioxidant activity compared to free drug. S-nitroso-N-acetylpenicillamine-derivatized generation-4 polyamidoamine dendrimers have shown therapeutic potential of reducing ischemia/reperfusion injury (Shcharbina et al. 2013).

12.4 Nanotechnology in Targeting Stroke Therapy

The movement of drug substances from the blood to the brain is regulated by BBB and the blood-cerebrospinal fluid barrier (BCSFB). However, BCSFB has an insignificant role in exchanging substances due to its inadequate method and much lesser surface area. Cells such as pericytes, endotheliocyte, and astrocytes form adherent junctions, tight junctions, the luminal surface-bound glycocalyx, and apicobasal polarity which limits blood-brain barrier permeability (Pivoriūnas and Verkhratsky 2021). Tight junction protein complexes between capillary endothelial cells include junctional adhesion particles, occludin, claudins, membrane-associated degrading enzymes, and membrane-associated guanylate kinase-like proteins.

Nutrient transport in the brain is regulated by efflux transporters such as breast cancer-related protein (BCRP), P-glycoprotein (P-gP), and multidrug resistance-associated proteins (MRPs). This altogether makes the restricted entry of substances into the brain. Two main nutrient transporters for glucose and L-type amino acid transporters, GLUT1 and LAT1, are expressed in brain capillaries and brain microvessel endothelial cells (BMEC), respectively. Other transporters include excitatory amino acid transporter 2 (EAAT2), monocarboxylate transporter 1 (MCT1), organic cation transporters (OCT), and organic anion transporters (OAT). Expression of these transporters has been taken into account before designing targeted drug delivery to the CNS (Wu et al. 2020).

Nanomaterials used in stroke therapy include carbon nanotubes, metal nanoparticles, liposomes, polymers, graphene, quantum dots, hydrogels, black phosphorus, and dendrimers (Fig. 12.4).

Nanotechnology overcomes hurdles associated with stroke such as ineffective drug delivery into the brain, by improving biodistribution, long circulation times, increasing solubility, extending half-life, reducing immunogenicity, controlling drug release, stability, doses due to the heterogeneous nature of the stroke (Fig. 12.5).

So, nanotechnology helps both in drug delivery and diagnostic (“theranostics”), in addition to its use in tissue engineering.

Nanotechnology is mainly used as a carrier system to deliver neuroprotective agents and anti-inflammatory drugs, block proinflammatory cytokines, reduce immune cell adhesion, and decrease cell apoptosis, lipid peroxidation, and small interfering RNA (siRNA), etc. (Wu et al. 2020). Applications of NPs in stroke depend on their size and the time of administration as their viscosity changes with size which further decreases hemoglobin and hematocrit concentration in addition to red blood cell deformability (Shcharbina et al. 2013).

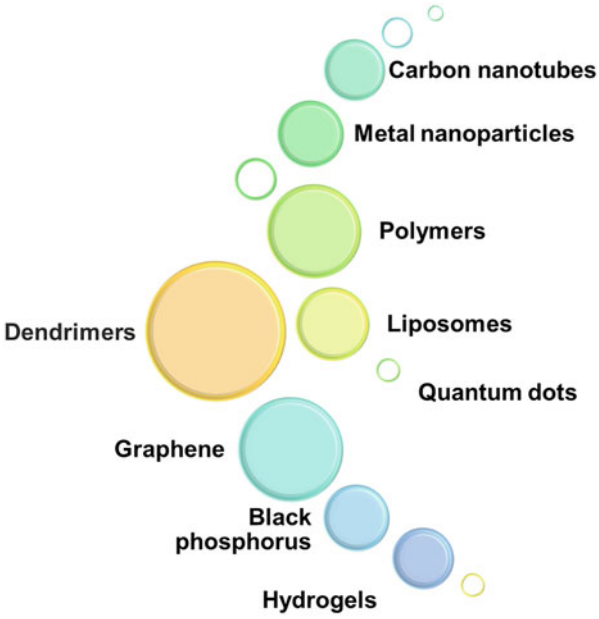

Fig. 12.4 Nanomaterials used in stroke therapy

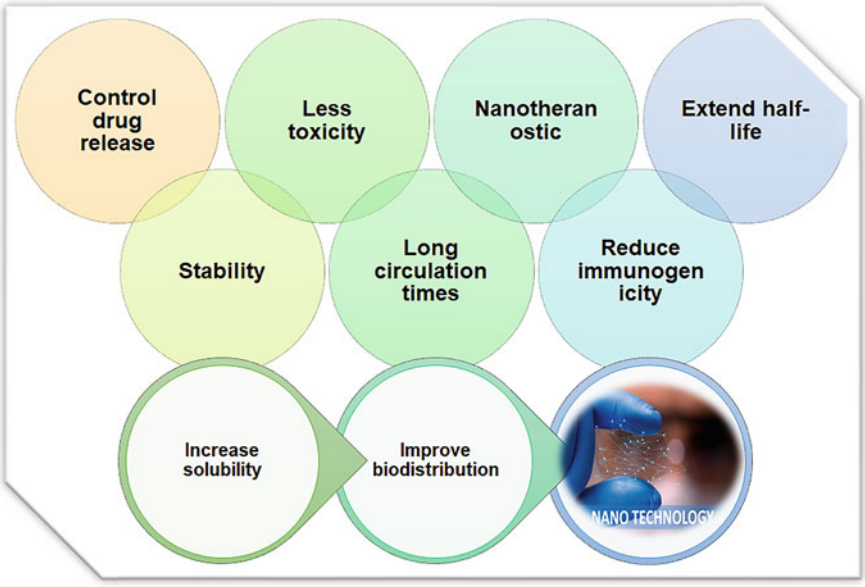

Fig. 12.5 Advantages of nanotechnology in stroke therapy

Therefore, the development and selection of suitable drug delivery vehicles are very important to make their use in public.

12.5 Nanocarriers Used in Stroke Therapy

12.5.1 Liposomes

Both hydrophobic and hydrophilic drugs can be loaded into liposomes due to their cell membrane-like structure that provides them with good biocompatibility, little toxicity, and extended circulation period. Moreover, they are also modified to targeted delivery and controlled release can be achieved due to their easy surface modification properties and lipophilicity and thus allowing them to enter BBB. An anti-inflammatory drug acetate prolonged the half-life and decline its toxicity when encapsulated within liposomes. Liposomes are considered perfect transporters for antibodies and imaging agents (Wu et al. 2020). However, these are used to deliver drugs, nucleic acids, vaccines, and proteins (Tian et al. 2021). Polyethylene glycol (PEG)-modified liposomes were used aimed at active drug delivery of mouse paired immunoglobulin-like receptor B (PirB). Citicoline was delivered into cytidine-5′-diphosphocholine (CDPC) captured liposomes employing vascular cell adhesion molecule-1 effectively improving local delivery. Enhanced signal intensities of nanoprobes were obtained upon near-infrared fluorophore 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) encapsulation in immunoliposomes (Wu et al. 2020).

Liposomes were used to deliver basic fibroblast growth factor (bFGF) inside the brain. The therapeutic effect of bFGF-NL is related to PI3K/Akt stimulation that reduced infarct volume, upgraded neurological function, and reduced nuclear fragmentation of neurons. Liposomal fasudil (fasudil-Lip) was combined with tPA increased neuroprotective effects and prolonged the therapeutic time window of tPA in comparison alone. Liposomal formulations prepared from HSPC/CHOL/DSPE-PEG2000 were selected to govern when drugs should be given before the reperfusion surgical treatment. PEGylated-liposomes have lengthy blood circulation and showed their accumulation even after 48 h of stroke. 3-n-Butylphthalide (dl-NBP) PEGylated-lipid nanoparticles (PLNs) coupled using Fas ligand was targeted to the ischemic region of the brain (Tuttolomondo et al. 2009).

12.5.2 Polymeric Nanoparticles

Polymeric nanoparticles have the ability to modify drug release, increase the stability of volatile drugs, and incorporate them into other activities related to drug delivery, with limitations such as high cost, the complex preparation process, and low the reproducibility. Polymeric nanoparticles loaded with Z-DEVD-FMK showed a significant decrease in nerve injury, caspase-3 activity, and reduced infarct volume in stroke. Cationic polymer micelles have high efficiency, safe and reliable for tracking stem cells in vivo using magnetic resonance imaging (Tian et al. 2021). Natural or synthetic polymers are used to construct nano-sized particles called

polymeric nanoparticles. Among studied NPs, chitosan and polylactic acid-glycolic acid copolymer (PLGA) have been verified to be effective drug delivery systems. Coating with the PEG lipid layer is used in order to raise blood half-life, reduce nonspecific binding, and allow precise ligand binding (Wu et al. 2020).

12.5.2.1 Chitosan

Chitosan is a polysaccharide that has been recognized as the second most abundant polysaccharide in nature which has shown its importance due to its natural origin and several biological properties such as biocompatibility, non-toxicity, non-allergenicity, and biodegradability, as well as its antifungal, antibacterial, antioxidant, anti-tumor and anti-inflammatory activities. Furthermore, it has immunoadjuvant, anti-thrombogenic, and anti-cholesteric properties. Its use in drug delivery, gene delivery, tissue engineering, and regenerative therapies is due to its versatile nature including it can be used in many physical forms such as fibers (and nanofibers), gels, sponges, beads, films, particles (and nanoparticles), membranes, and scaffolds (Ojeda-Hernández et al. 2020). Gallic acid (GA), miRNA-124, imipramine, C-Phycocyanin-pertaining liposome (C-Pc liposome), etc. are some of the chitosan-based treatment strategies been tried in preclinical experiments involving stroke (Aderibigbe and Naki 2019). Peptide inhibitors for caspases (e.g., N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (Z-DEVD-FMK)) were developed into chitosan nanospheres.

12.5.2.2 Dendrimer

Dendrimers are branching polymers whose structure becomes globular and densely packed at the periphery as generation increases. Dendrimers possess some properties like monodispersity, functional end groups that are responsible for their high solubility and reactivity hence also used in contrast agents for MRI, new synthetic vectors for gene therapy, drug-delivery carriers, drugs for the treatment of prion diseases, and neurodegenerative disorders. In the treatment of stroke, dendrimers are the second prospective candidates, showing themselves to be effective carriers of heparin in preventing deep vein thrombosis in a rodent model (Shcharbina et al. 2013). Dendrimer-based albumin nanoparticles, polyamidoamine as a gene carrier, cationic poly(amido amine) (PAMAM) functionalized with poly(ethylene glycol) (PEG), salvianic acid A, etc. are some of the treatment strategies tried in preclinical studies involving stroke. Polyamidoamine (PAMAM) dendrimer is used for gene delivery. PAMAM dendrimers were amide embedded with basic L-arginine residues (e-PAM-R) used to transport the great motion group box-1 (HGMB1) siRNA that showed a substantial reduction in neuronal cell decease and HMGB1 levels (Kim et al. 2010).

12.5.3 Bioengineered Nanoparticles

12.5.3.1 ROS-Responsive NPs

Drug release was controlled using ROS-responsive bioengineered nanoparticles. Polymer nanoparticle employing ROS-responsive boronic ester was developed with self-assembling red blood cell (RBC) membrane then named SHp-RBC-NPs.

A peptide, SHp (CLEVSRKNC), coupled NPs can control the release of NR2B9C which is activated by increased levels of ROS that prevented neural damage and reduced the brain infarction magnitude (Lv et al. 2018).

12.5.3.2 Protease-Responsive NPs

Protease-responsive AMD3100 (a targeting ligand)-conjugated and size-shrinkable nanoparticles (ASNPs) were established to target the ischemic brain (Guo et al. 2018).

12.5.4 Biomimetic Nanoparticles

Better biosafety and advanced targeting abilities make the preference of cell-derived biomimetic carriers over artificial carriers. Cell membrane-derived nanovesicles were used to target activated endothelium.

12.5.4.1 Erythrocyte Membrane Nanovesicles

Erythrocyte membrane nanovesicles laden using Mn3O4 nanoparticles (Mn3O4@nanoerythrocyte-T7, MNET) linked T7 peptide (a brain targeting peptide) used to change hypoxia environments and scavenge free radicals (Shi et al. 2019).

12.5.4.2 Platelet Membrane-Derived Nanovesicles

L-arginine and γ-Fe2O3 magnetic nanoparticles (PAMNs) were formulated with platelet membrane-derived nanovesicles used for both targeted therapy and MRI imaging purposes (Li et al. 2020).

12.5.4.3 Exosomes

Exosomes are endogenous vesicles made from cell membranes used as drug delivery agents because of their innate stability, biodegradability, ability to cross BB, and low immunogenicity. In stroke, exosomes were found to reduce inflammation and increase angiogenesis, neurogenesis, and white matter remodeling. They are obtained from cells including MSCs and their modified form are used as vehicles to transport exogenous genes, proteins, and chemical compounds to recipient cells (Chen and Chopp 2018). In stroke, exosomes showed restorative therapeutic effects by mediating cell–cell communication and may improve stroke by regulating nervous system, remodeling of blood vessels and inhibiting neuroinflammation.

12.5.4.4 Mesenchymal Stromal Cell (MSC)

Mesenchymal stromal cell (MSC)-derived exosomes were used to deliver curcumin after conjugation through a c(RGDyK) peptide (called cRGD-Exo) (Zhu et al. 2019).

12.5.4.5 R3V6 Peptide (with a 3-Arginine Block and a 6-Valine Block)

R3V6 peptide (with a 3-arginine block and a 6-valine block) incorporated with dexamethasone formed firm micelle (called, R3V6-Dexa) used intended for gene transfer (Lee et al. 2012; Fig. 12.6).

12.5.5 Inorganic Nanoparticles

Inorganic/metallic NPs function as ROS scavengers. Their size and shape can easily be controlled during synthesis, their surface is also easy to modify, and hence their quality requirements for clinical applications can be met. They have unique surface plasmon resonance characteristics, enhanced photothermal capability, and thus a great likelihood for treatment and recognition. Magnetic metal nanoparticles can be used meant for magnetic targeting, magnetic resonance imaging (MRI), and magnetic hyperthermia (Naqvi et al. 2009).

12.5.5.1 Ceria Nanoparticles (E-A/P-CeO2)

Ceria nanoparticles react reversibly utilizing oxygen to Ce4+ (oxidized) species after Ce3+ (reduced) type and prevented apoptosis, shielding the brain from ischemic injury. The limitation of BBB with ceria nanoparticles (E-A/P-CeO2) was overcome by modification with poly(ethylene glycol) and angiopep-2 (ANG) (Bao et al. 2018).

12.5.5.2 Platinum Nanoparticles (nPt)

Platinum nanoparticles (nPt) were also used as ROS scavengers in I/R injury because of their high electron density and large surface area and suggested to be joint with tPA reperfusion management to manage ischemic stroke patients (Dong et al. 2020). Superparamagnetic properties of iron oxide nanoparticles protect materials from self-aggregation.

Fig. 12.6 Various nanostructures used as delivery vehicles and their applications in stroke

12.5.5.3 Carbon Nanotubes (CNT)

Carbon nanotubes are there composed of graphite sheets tubes that display single- or multi-walled structures, described by exposed ends or locked with fullerene lids. CNTs function to encourage active transport to the brain. However, the result of fullerenes and CNT on neuroprotection at this step is still indefinite. They can encourage blood coagulation; hence, their application in stroke requires significant surface modification. Carbon nanotubes turn into stem cell treatment, and their functionalizing might improve their therapeutic probable for stroke action (Naqvi et al. 2020).

12.5.5.4 Black Phosphorus

Black phosphorus (BP), namely, phosphorene, has a wide-ranging spectrum of intense light captivation used in the arena of photoacoustic imaging, photothermal treatment, fluorescence imaging, etc. BP showed great BBB penetrability under near-infrared irradiation (Qiu et al. 2018a). Black phosphorus (black P) was recently rediscovered from the perspective of a 2D- layered material thus promising for novel applications in nanoelectronics and nanophotonics (Ling et al. 2015). Black phosphorus (BP) nanosheets (BPNSs) and BP quantum dots (BPQDs) have already been used for bioimaging, photothermal therapy, photodynamic therapy, and drug delivery platforms (Qiu et al. 2018).

12.5.6 Hydrogels

Hydrogels contest the mechanical properties of the ordinary brain offering structural support which is used to repair injured tissue. Injectable hydrogels can successfully circumvent the blood-brain barrier for carrying neural progenitor cells. Accumulation of intracranial pressure during hydrogel injection using noninvasive imaging is prevented using magnetic resonance imaging to monitor the injection. Osteopondin was encapsulated with gelatin microspheres (GMS) showing increased neuroprotection, decreased inflammation, and extended duration of osteopondin release significantly (Jin et al. 2014). Unremitting and progressive delivery of epidermal growth factor (EGF), erythropoietin, and cyclosporine A was achieved using hydrogel.

12.5.7 Lipid Nanofomulations

Nanostructured lipid carriers (NLCs) and solid lipid nanoparticles (SLNs) are made from fatty ingredients so they are used to load lipophilic drugs and may possess lengthy retention time and high oral bioavailability. Lipid NPs are used to cross the BBB even without any surface modification.

12.5.7.1 Nanostructured Lipid Carriers (NLCs)

Sesamol was incorporated into the nanostructured lipid carriers (S-NLCs) and its therapeutic potential was evaluated in in vitro and in vivo models of the ischemic

stroke where it showed prolonged protective effects via activation of PI3K signaling pathway (Hassanzadeh et al. 2017). It is speculated that nimodipine (NMD) may improve the effect of NMD in stroke treatment (Liu et al. 2021). Temazepam (gamma-aminobutyric acid (GABA) modulator)-loaded nanostructured lipid carriers (NLCs) have shown possibilities for enhancing bioavailability and brain targeting affinity after oral administration (Eleraky et al. 2020). A lipid-based nanotherapeutic vector composed of biomimetic lipids and CeO_2 nanoparticles has been shown to have an inherent ability to cross the blood-brain barrier (Battaglini et al. 2019). The bioavailability of ferulic acid (FA) was enhanced after loading into NLC (Wu et al. 2020).

12.6 Nanoparticles Targeting Receptors in Stroke

12.6.1 EPO Receptor (EPOR)

Asialoerythropoietin (AEPO) is a neuroprotective manager that is modified with PEGylated liposomes (AEPO-liposomes) which fixes to EPO receptor (EPOR) on neural cells and gets accumulated in the ischemic lesion. AEPO-liposomes activate MAPK and PI3K/Akt pathways, reduce neuronal apoptosis, decrease infarct lesions, and ameliorate cerebral I/R damage in rats.

12.6.2 Transferrin Receptor (TfR)

ZL006 conjugated with HAIYPRH (T7), loaded into PEGylated liposomes (T7-P-LPs) can selectively block the ischemia-induced binding between PSD-95 (a scaffolding protein) and nNOS (neural cell nitric oxide synthase) thus making the BBB transport of ZL006. T7 improved the transportation of liposomes through the blood-brain barrier; T7-PLPs/ZL006 pointedly concentrated infarct size and prohibited neurological insufficiency. Therefore, an alternative dual targeting delivery structure using T7 peptide and stroke homing peptide (SHp, CLEVSRKNC)-conjugated liposome (T7 & SHp-P-LPs/ZL006) was developed which lessened the infarction sizes in the brain and improved neurological impairment (Zhao et al. 2016).

12.6.3 CXCR4 (C-X-C Chemokine Receptor Type 4)

Ischemic brain material is enriched with C-X-C chemokine receptor type 4, and thus glyburide nanoparticles were developed to target this area.

12.6.4 Low-Density Lipoprotein (LDL) Receptors

BBB overexpresses low-density lipoprotein (LDL) receptors and thus apolipoproteins absorbing SLNs or NLCs which are then actively transported into

the brain utilizing receptor-mediated endocytosis. Resveratrol-loaded SLN with apolipoprotein E (ApoE) possibly will cross the blood-brain barrier further professionally compared to non-functionalized SLN.

12.6.5 Lactoferrin Receptors (Fig. 12.7)

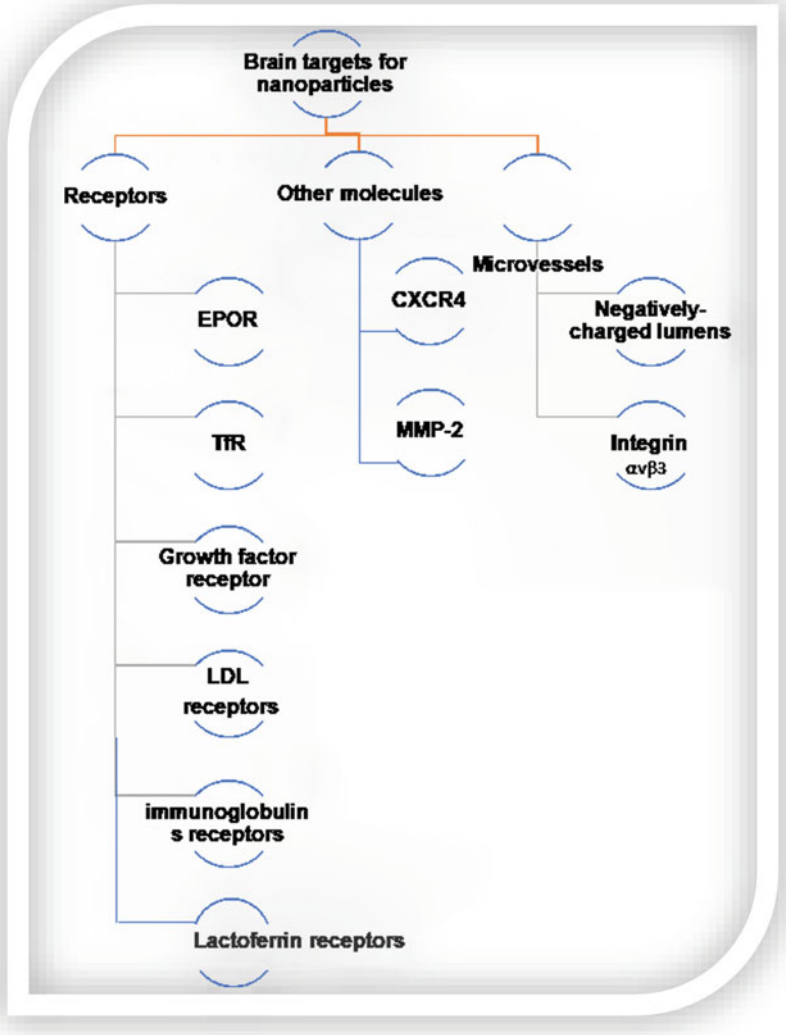

Fig. 12.7 Nanoparticles targeting receptors in the brain

12.7 Nanoparticles Targeting Brain Microvessels

Negatively indicted lumens of brain microvessels were targeted using tanshinone IIA PEGylated nanoparticles (CBSA-PEG-TIIA-NPs) coupled utilizing cationic bovine serum albumin (CBSA). The mechanism involved was investigated and significantly suppressed the inflammation response, abridged the infarcted volume and neutrophil infiltration, prohibited microglial activation, and prevented the neuronal apoptosis.

12.7.1 Integrin αvβ3

With the ischemic injury to the brain, integrin αvβ3 is selectively upregulated, and thus, inhibition of integrin αvβ3 preserves microcirculation. Furthermore, the vascular endothelial growth factor is a known inducer of integrin αvβ3 expression. Thrombin has also been shown to interact with integrin αvβ3 by inhibiting the deposition of fibrinogen. The integrin αvβ3 inhibitor cRGDfV improves outcomes in the MCAO model (Shimamura et al. 2006). c(RGDyK) peptide has a high affinity for integrin αvβ3 thus used to deliver curcumin that repressed the inflammation via the NF-κB pathway (Dong et al. 2020).

12.8 Limitations and Future Perspectives

Stroke involves activation of several cascades that gives a very short window to get therapeutic intervention mediated recovery. It also required the adoption of multi-targeted drug discovery and delivery to the ischemic area. The discovery of new agents is necessary as existing drugs have their limitations including non-recovery of degenerated neurons and no single neuroprotective mechanism is generally effective. Selecting a preclinical stroke model that should adequately emulate the clinical condition is one of the important steps in animal research. The scarcity or absence of small-molecule blockers of cation ion channel families, dose, blood-brain barrier permeability, and period of administration are some of the factors that should be taken into consideration while selecting antioxidant therapy in stroke. Efflux transporters and various junctions in BBB have to be taken into consideration while designing novel therapeutic targets (Fig. 12.8).

Although nanotechnology overcomes the limitations associated with drugs, it has its challenges such as the development of a novel drug delivery system, toxicity, etc. which have to be taken into consideration. Injected particles cause immune activation and are rapidly cleared as they are recognized as pathogens by the host than soluble molecules. Also, it may activate thrombosis-associated pathology including the kallikrein-kinin system or the contact activation pathway and activation of platelets which may become toxic when entering tissues. Mononuclear phagocyte system sequesters majority of intravenously injected nanoparticles which might disrupt organelles such as lysosomes or endoplasmic reticulum or mitochondria,

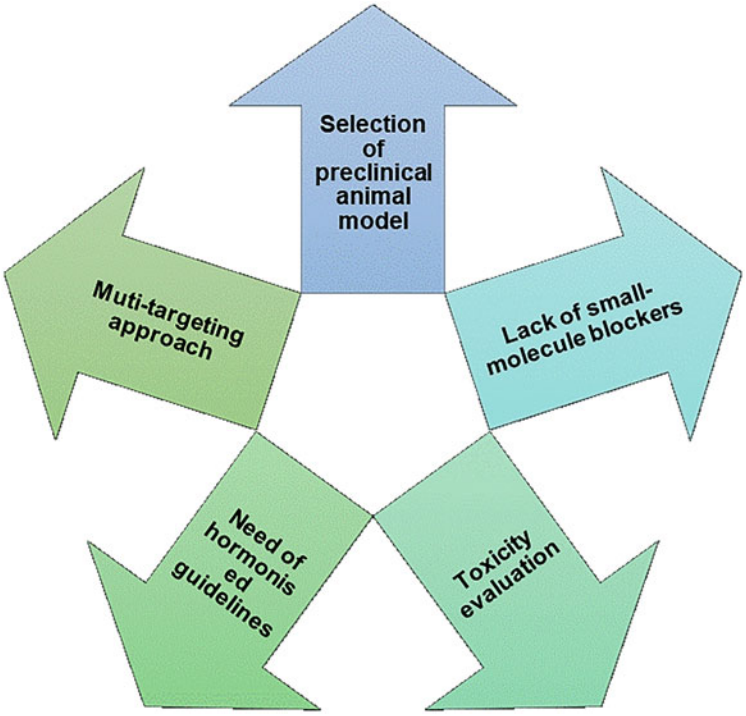

Fig. 12.8 Future perspectives are taken into consideration for stroke therapy

which leads to production of excess reactive oxygen species and further release of proinflammatory mediators inside macrophages. The cell nucleus may be exposed to NPs and cause DNA damage; therefore, it requires genotoxicity assessment (Fig. 12.9).

New technologies have to be developed to scale up cell membrane-derived nanovesicles. Targeting inflammatory neutrophils is one of the new strategies used to treat ischemic stroke. Nanoparticle therapeutics should be started in the desired time window after the occurrence of the stroke to give the fastest drug therapy. Nanoparticle-based platforms are of great utility in theranostic purposes as they allow constructing more than one imaging agents and drugs in a single nanoparticle. Contrast agents used in imaging should possess ideal characteristics of long-circulating half-life, a contrast to noise enhancement, sensitivity, nontoxicity, selectivity against epitope, and capability to be imaged with commercially obtainable equipment.

Numerous inorganic nanoparticles do not exist obviously in the body; hence, toxicity evaluation is required to get their FDA approval. There are no harmonized guidelines for the nanoparticle safety evaluation, but the Food and Drug Administration and the European Union have adopted specific schemes for the approval and legislation of nanopharmaceuticals.

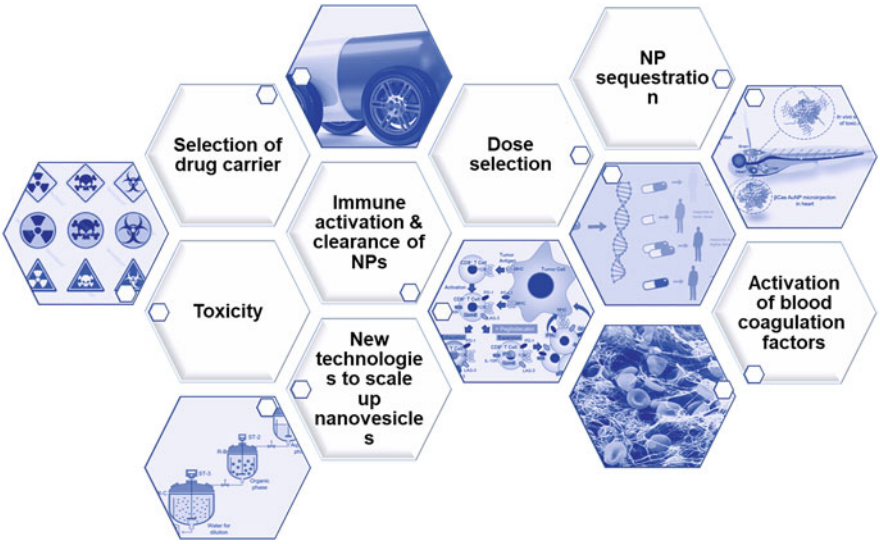

Fig. 12.9 Limitations of nanotechnology used in stroke therapeutics

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Conflicts of Interest The authors declare no conflict of interest.

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Signaling Pathways of Interest for Enhancing Recovery from Ischemic Stroke

13

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13.1 Introduction

Patients recovering from ischemic stroke remain at risk for subsequent ischemic events. This is because multiple signaling molecules that are risk factors for stroke can themselves get upregulated as a result of vascular and neural injuries caused by ischemic stroke. Consequently, it is important to disrupt this vicious cycle of inflammation and stroke by enhancing repair mechanisms such as clot clearance, neurogenesis, and anti-inflammatory response. By discussion of various biomolecules and pathways involved in these mechanisms, this chapter aims to provide the foundational analysis necessary for comprehensive guidance of future research and therapeutic efforts in a neuro-restoration amongst stroke patients. These mechanisms shall be explored in two distinct sections, viz., (1) "Mechanisms of Clot Formation" and (2) "Enhancement of Neurogenesis."

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13.2 Mechanisms of Clot Formation

13.2.1 Platelet Activation and Aggregation

Platelets are an integral component in the production of thrombus and primary hemostasis. Upon coming into contact with exposed collagen at sites of vascular injury, they become activated which leads to their aggregation. This forms a platelet plug which seals off the injured area as shown in Fig. 13.1 (Rivera et al. 2009; Periayah et al. 2017). Platelet adherence to the subendothelial surfaces is aided by a variety of proaggregatory stimuli, also known as platelet agonists. Platelets change shape, discharge their granule contents, and gradually form aggregates by sticking to one another during this process (Cerletti et al. 2012; Vinik et al. 2001).

13.2.2 Coagulation Cascade

Coagulation takes place due to a series of proteolytic events involving the sequential activation of coagulation factors. There are two different mechanisms, the extrinsic or tissue injury pathway and the intrinsic or contact pathway; both may activate the subsets of thrombogenic components (Chaudhry et al. 2020). The liver is responsible for synthesizing and secreting blood coagulation components. Fibrinogen; prothrombin; factor V, VII, IX, X, XI, and XII; protein C; protein S; and antithrombin are produced by hepatocytes in the liver, while factor VIII and von Willebrand factor are produced by liver sinusoidal endothelial cells, as shown in Fig. 13.2.

Contact between the bloodstream and tissue factor activates un-activated factor VII to activated factor VIIa-tissue factor (VIIa-TF) which converts factor IX to IXa. The activated IXa in the presence of VIIIa causes conversion of X to Xa. Further cascade is carried in the presence of Va which converts prothrombin to thrombin (Wiggins and Cochrane 1979). Thrombin/coagulation factor IIa then cleaves fibrinogen (FI) to produce fibrin monomers (FIa). Coagulation factor XIIIa (FXIIIa) crosslinks these monomers to produce a fibrin clot as shown in Fig. 13.2 (Grover and Mackman 2019).

Feedback Mechanism for Regulating Coagulation

Thrombin/coagulation factor IIa activates coagulation factor V (FV), FVIII, protein S, and protein C in addition to directly creating active fibrin and FXIIIa.

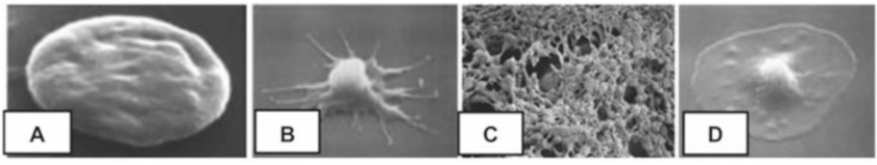

Fig. 13.1 (**a**) Platelet in resting mode. (**b**) Activated platelets change into a pseudopodia shape. (**c**) Aggregated platelets. (**d**) Platelet spreading. (Source: Periayah et al. 2017)

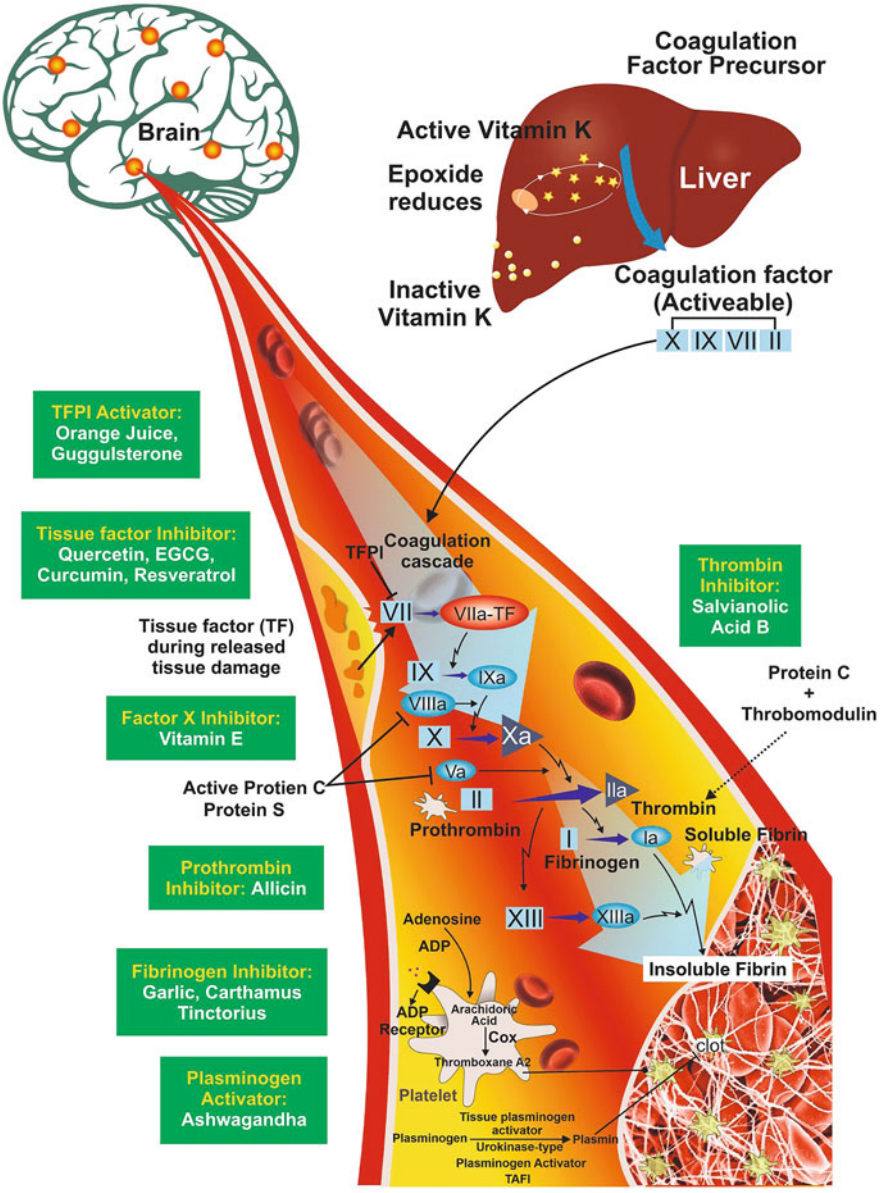

Fig. 13.2 Coagulation cascade

Through feedback control, these elements increase or decrease thrombin production. Coagulation factors Va and VIIIa promote thrombin formation by positively regulating either prothrombin cleavage or FX activation. Thrombin binding to thrombomodulin activates protein C, which results in the degradation of FVa and

Table 13.1 List of dietary supplements targeting biomolecules involved in coagulation

Target biomolecule	Compound	Activity	Type of study	References
Tissue factor	Quercetin	Inhibition	In vitro	Guglielmone et al. (2012)
	Epigallocatechin 3-gallate	Inhibition	In vitro	Wang et al. (2010)
	Curcumin	Inhibition	In vitro	Pendurthi et al. (1997)
	Resveratrol	Inhibition	In vitro	Di Santo et al. (2003)
Thrombin	Salvianolic acid B	Inhibition	In vitro	Xu et al. (2015)
Factor X	Vitamin E	Inhibition	In vivo (human)	Booth et al. (2004)
Prothrombin	Allicin	Inhibition	In vivo (human)	Rajaram (2003)
Fibrinogen	Garlic	Inhibition	In vivo (human)	Allison et al. (2012)
	Carthamus tinctorius	Inhibition	In vitro	Memariani et al. (2018)
Plasminogen	Ashwagandha	Activation	In vitro	Chen et al. (2015)
TFPI	Orange juice	Activation	In vivo (human)	Napoleone et al. (2013)
	Guggulsterone	Activation	In vitro	Gebhard et al. (2009)

FVIIIa and the regulation of prothrombin cleavage. Likewise plasminogen in the presence of tissue plasminogen activator and urokinase type plasminogen activator TAFI converts to plasmin which degrades fibrin clot (Grover and Mackman 2019; Gailani and Renné 2007). The blood coagulation cascade can be better controlled using feedback regulation and sequential activation of clotting components. This strict management is necessary to prevent uncontrolled blood loss caused by insufficient clotting or stroke due to excessive clotting. Several active constituents (Table 13.1) are known to inhibit various biomolecules involved in the coagulation cascade. Food products containing these active constituents may be incorporated into the dietary and supplement plan to better prevent stroke recurrence.

13.2.3 Role of Inflammation in Stroke

Ischemic stroke and associated brain injuries appear to have a strong link to inflammation. Systemic inflammation increases patients' susceptibility to stroke and its prognosis (Esmon 2000; McColl et al. 2009). Elkind et al. found that stroke patients who had systemic inflammation had worse clinical outcomes (McColl et al. 2007). Induced focal cerebral ischemia in an experimental cell line model triggered mobilization of inflammatory cells, namely, neutrophils, T cells, and monocytes/macrophages, whereas suppressing the inflammatory mechanisms reduced infarct

size and neurological impairment (Elkind et al. 2004). In vivo studies have also reported anti-inflammatory therapy to be effective, but attempts to convert this into clinical practice have failed. Thus, the activation of inflammatory cascades after focal cerebral ischemia-reperfusion (I/R) and their contribution to ischemic brain injury need to be thoroughly understood to develop more effective therapeutic interventions for the treatment of acute ischemic stroke (Prestigiacomo et al. 1999).

The complicated mechanisms of inflammation following stroke involve multiple phases of pro-inflammatory reactions. Determining the levels of cytokines during the acute phase of stroke has helped researchers understand the immune response seen during brain ischemia. Further research would aid in identifying interactions between the immune system and brain cells in the recovery and regeneration process after a stroke. A detailed understanding of inflammatory mechanisms can help in modifying immune response to reduce brain tissue damage and the risk of further clotting events.

Tissue Factor-Mediated Signaling: Since TF mimics a cytokine class II receptor, researchers have worked hard to show that FVIIa:TF causes signal transduction. In a variety of cell types, the creation of the complex does, in fact, cause phospholipase C-dependent calcium transients as well as activation of the p42/p44 mitogen-activated protein kinase (MAPK). Other MAPK isoforms, such as p38 MAPK and c-Jun N-terminal kinase (JNK), are activated depending on the cell type. FVIIa has also been shown to activate the anti-apoptotic kinase c-Akt/PKB in a variety of cell types. Finally, in keratinocytes, FVIIa:TF triggered by nuclear factor (NF)-B causes an increase in the coagulation cascade (Jin et al. 2010; Li et al. 2009).

IL-6: Interleukin-6 (IL-6) mediates a range of platelet responses, including thrombocytosis, platelet hyper-reactivity, and accelerated thrombus formation (Ishibashi et al. 1989; Burstein 1994). IL-6 with its receptor gp130 has been shown to activate megakaryocytes (MPL) via STAT3 and thrombopoietin (TPO) to increase platelet production as depicted in Fig. 13.3 (Burstein 1994; Peng et al. 1994).

TNF-α: Tumor necrosis factor α (TNFα) activates an adapter protein called TNF receptor-associated death domain (TRADD), which then activates TNFR-associated factor 2 (TRAF2). It is also known to activate NFκβ (nuclear factor-kappa beta), which binds to the IKK complex and releases pro-inflammatory cytokines including TNF-α and IL-6, resulting in tissue factor activation (Peng et al. 1994).

IL-1β: IL-1β activates translocating chain-associated membrane protein (TRAM), which then activates adaptor protein myeloid differentiation factor 88 (MyD88) and leads to downstream activation of TNF receptor-associated factor 6 (TRAF6) via the IRAK1 and IRAK4 proteins of the IL-1 receptor-associated kinase (IRAK) family. Through protein kinase B (Akt) and JNK, TRAF6 activates

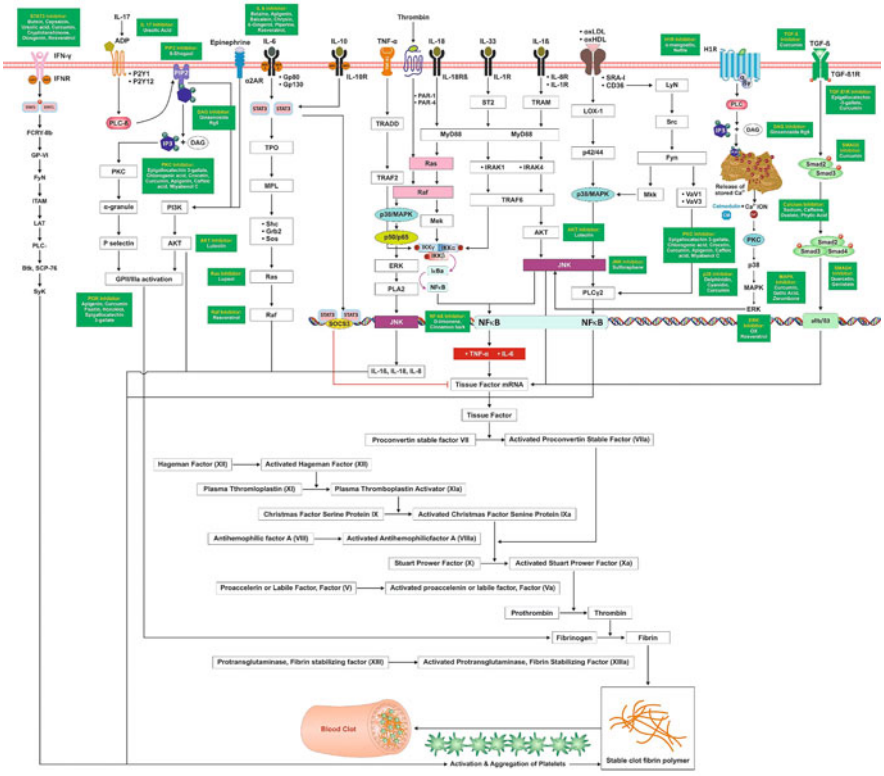

Fig. 13.3 Role of cytokines in clot formation

NFκβ. Other pro-inflammatory cytokines (TNF-α, IL-6) involved in the coagulation cascade are produced due to NFκβ activation, and these cytokines are involved in the coagulation cascade (Mutlu et al. 2007).

Other pro-inflammatory signaling molecules that activate pathways leading to increased clotting include histamine receptor H1, epinephrine, thrombin, IL-13, IL-17, IL-18, IL-33, interferon gamma (IFN-γ), and TGF-β, as well as oxidized LDL and HDL, as depicted in Fig. 13.3. These molecules and their downstream pathways are inhibited by several active constituents (Table 13.2). The inclusion of these active constituents in the diet plan of stroke patients may help mitigate the inflammatory cascades contributing toward risk of stroke recurrence and further tissue damage (Senchenkova et al. 2013; Pircher et al. 2012; Xie et al. 2015; Fujisawa et al. 2012; Cui et al. 1996; Guha and Mackman 2002; Lang et al. 2003; Lindmark et al. 1998; Miyoshi et al. 2008; Schmitz et al. 2005; Adderley et al. 2015).

Table 13.2 List of dietary supplements targeting interleukins involved in coagulation cascade

Target biomolecule	Compound	Activity	Type of study	References
STAT3	Butein	Inhibition	In vitro	Trécul et al. (2012)
	Capsaicin	Inhibition	In vitro	Bhutani et al. (2007)
	Ursolic acid	Inhibition	In vitro	Trécul et al. (2012)
	Cryptotanshinone	Inhibition	In vitro	Shin et al. (2009)
	Curcumin	Inhibition	In vitro	Bharti et al. (2003)
	Diosgenin	Inhibition	In vitro	Li et al. (2010)
	Resveratrol	Inhibition	In vitro	Baek et al. (2016)
IL-17	Ursolic acid	Inhibition	In vivo (mice)	Xu et al. (2011)
	Luteolin	Inhibition	In vitro	Zhang et al. (2016a)
PIP2	6-Shogaol	Inhibition	In vitro	Townsend et al. (2014)
RAF	Resveratrol	Inhibition	In vitro	Guerra and Issinger (2019)
RAS	Lupeol	Inhibition	In vitro	Saleem (2009)
JNK	Sulforaphane	Inhibition	In vitro	Subedi et al. (2019)
DAG	Ginsenoside Rg5	Inhibition	In vitro	Xiao et al. (2017)
IL-6	Betaine	Inhibition	In vitro	Xia et al. (2018)
	Apigenin	Inhibition	In vitro	Qiu et al. (2019)
	Baicalein	Inhibition	In vitro	Jelić et al. (2016)
	Chrysin	Inhibition	In vitro and in vivo (Chick)	Lin et al. (2010a, b)
	6-Gingerol	Inhibition	In vitro	Li et al. (2013)
	Piperine	Inhibition	In vitro	Pradeep and Kuttan (2004)
	Resveratrol	Inhibition	In vitro	Akhondzadeh et al. (2020)
NFκB	D- limonene	Inhibition	In vivo (mice)	Babaeenezhad et al. (2021)
	Cinnamon bark	Inhibition	In vitro	Schink et al. (2018)
PKC	Epigallocatechin 3-gallate	Inhibition	In vitro	Kitano et al. (1997)
	Chromogenic acid	Inhibition	In vitro	Das et al. (2016)
	Crocetin	Inhibition	In vitro	Wang et al. (1996)
	Curcumin	Inhibition	In vivo (rats)	Soetikno et al. (2012)
	Apigenin	Inhibition	In vitro	Huang et al. (1996)
	Caffeic acid	Inhibition	In vitro	Szaefer et al. (2007)
	Miyabenol C	Inhibition	In vitro	Kulanthaivel et al. (1995)
H1 receptor	α-Mangostin	Inhibition	In vitro	Chairungsrilerd et al. (1996)
	Nettle	Inhibition	In vitro	Roschek et al. (2009)
TGF beta	Curcumin	Inhibition	In vitro	Thacker and Karunagaran (2015)

(continued)

Table 13.2 (continued)

Target biomolecule	Compound	Activity	Type of study	References
TGF beta 1	Epigallocatechin-3-gallate	Inhibition	In vitro	Hsieh et al. (2017)
	Fisetin	Inhibition	In vitro	Shon et al. (2016)
Smad 2	Quercetin	Inhibition	In vitro	Cai et al. (2018)
Smad 4	Quercetin	Inhibition	In vitro	Cai et al. (2018)
	Genistein	Inhibition	In vitro	Phuah and Nagoor (2014)
ERK	Oxyresveratrol	Inhibition	In vitro	Chen et al. (2013)
P38	Delphinidin	Inhibition	In vitro	Lin et al. (2017)
	Cyanidin	Inhibition	In vitro	Lin et al. (2017)
	Curcumin	Inhibition	In vivo	Epstein et al. (2010)
MAPK	Curcumin	Inhibition	In vivo	Xiao et al. (2018)
	Gallic acid	Inhibition	In vivo	Park (2011)
	Zerumbone	Inhibition	In vivo	Li et al. (2020)
Calcium	Caffeine	Inhibition	In vivo (Human)	Hasling et al. (1992)
	Oxalate	Inhibition	In vivo (Human)	Milman (2020)
	Phytic acid	Inhibition	In vivo (Human)	Bohn et al. (2008)
	Sodium	Inhibition	In vivo (Human)	Teucher et al. (2008)

13.3 Enhancement of Neurogenesis

Neurogenesis is the formation of new neurons in the brain when neural precursor cells proliferate, differentiate, and migrate. During the process of embryonic development, neurogenesis brings about brain development and continues in specific regions of the brain in adults, namely, the lateral subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus (Pang et al. 2019). It has been observed that not only intrinsic factors like neurotrophic growth factors, neurotransmitters, cytokines, and hormones but also extrinsic factors like lifestyle play a role in stimulating and regulating neural stem cell proliferation and central nervous system development (Hollands et al. 2017).

13.3.1 Role of Neurotransmitters in Neuroregeneration

Neurogenesis in adults has repeatedly been demonstrated to occur in SVZ and SGZ regions of the adult human hippocampus. Neurotransmitters are among the main factors that help neuroblasts proliferate, differentiate, migrate, and survive in the SVZ and SGZ (Niklison-Chirou et al. 2020). Studies suggest that ablation of their receptors causes inhibition of neurogenesis and reduction of neuroplasticity of neurons (Borta and Höglinger 2007). Therefore, their role in the neuro-restorative

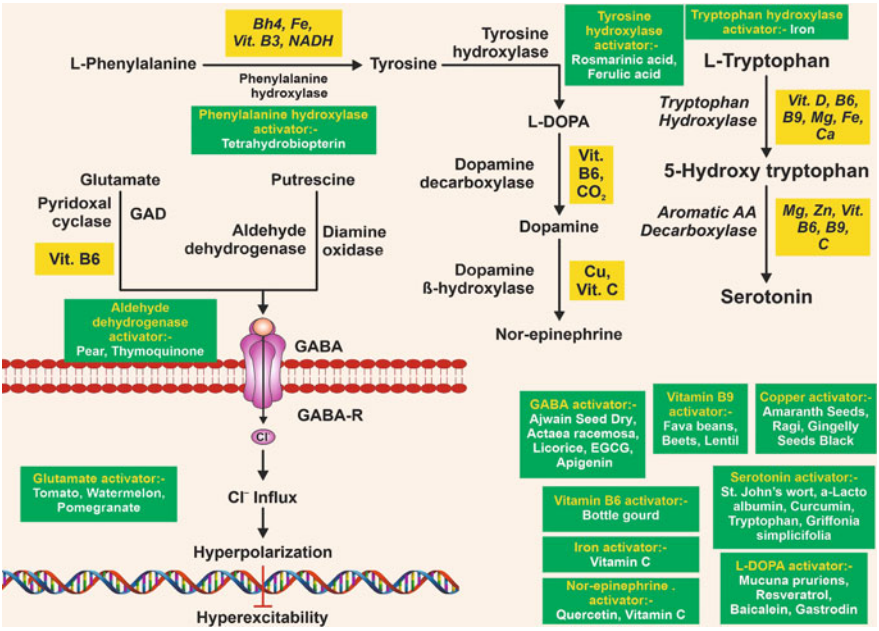

Fig. 13.4 Biosynthesis and significance of neurotransmitters

process needs to be explored to improve recovery in ischemic stroke patients. An understanding of the biosynthesis of each neurotransmitter is also required to improve their availability in such patients.

1. **GABA (gamma-Aminobutyric Acid)**

   Biosynthesis: Glutamate can easily cross the blood-brain barrier and get converted into GABA in the brain. Synthesis of GABA in the cytoplasm of presynaptic neurons from glutamate is brought about by the enzyme glutamate decarboxylase (GAD) with the involvement of pyridoxal cyclase as a cofactor. Alternatively, diamine oxidase and aldehyde dehydrogenase are two other enzymes that can synthesize GABA from putrescine (Ilie et al. 2012; Chattopadhyaya et al. 2007; Kirk and Richardson 1995; Bai et al. 2015).

   Significance: In the human brain, gamma-aminobutyric acid (GABA) is the neurotransmitter with a predominant inhibitory role. GABA also activates certain pathways that lead to dendrite arborization and spine stability that improves memory. Dysfunctional GABA signaling is associated with depression, anxiety, cognitive impairment, and defects in synaptogenesis (Calderón-Ospina and Nava-Mesa 2020). GABA binds to its receptor GABAAR to initiate chloride influx thus leading to hyperpolarization to control hyperexcitability (Calvo-Flores Guzmán et al. 2018; Fig. 13.4).

2. **Norepinephrine**
   Biosynthesis: Dopamine is converted into norepinephrine (NE) by the enzyme dopamine beta hydroxylase with copper and vitamin C as cofactors (Rahman et al. 2009). NE is mainly synthesized in the subcortical region of the brainstem called locus coeruleus (LC). LC is the small nucleus present in the posterior region of pons. This brain region controls diverse functions like cognition, homeostasis, motor behavior, sensory processing, learning, and memory (Moret and Briley 2011).
   Role: NE has both synaptic release and nonsynaptic release sites. The clusters of neurons in the LC that contain NE have widespread neuraxis and are the only source of NE to the various key parts of the brain such as hippocampus, thalamus, neocortex, etc. The LC-NE system is involved in various types of mental and physiological processes. Changes in the LC-NE system have been reported in many neurological diseases related to impaired cognition, mood disorders, and neurodegeneration (Moret and Briley 2011). Various preclinical studies have shown that restoring NE function could have a great potential to reduce neurodegeneration by increasing anti-inflammatory mechanisms and decreasing the inflammatory cascades. Norepinephrine is also involved in the expression of brain-derived neurotrophic factor (BDNF) through the phosphorylation of cAMP-response element binding (CREB) protein (Chen et al. 2007).
3. **Serotonin**
   Biosynthesis: L-tryptophan, an essential amino acid, is the precursor molecule which gets converted to serotonin by two sequential reactions. First, tryptophan hydroxylase in the presence of cofactors (viz., vitamin D, B6, B9, magnesium, calcium, and iron) converts L-tryptophan to 5-hydroxytryptophan (5-HTP). Next, aromatic L-amino acid decarboxylase (AAAD) in the presence of its own co-factors (magnesium, zinc, vitamin B6, B9, and C) converts 5-HTP to 5-hydroxytryptamine (5-HT), i.e., serotonin (Höglund et al. 2019).
   Role: Serotonin has the highest number of receptors when compared with other neurotransmitters. Consequently, it plays a role in a variety of neurological functions. Serotonergic signaling plays many important roles in the regulation of neuronal outgrowth, neuron survival, and synaptogenesis. Fanibunda et al. reported serotonin increased mitochondrial biogenesis in rodent cortical neurons by increasing the expression of mitochondrial components. This is important as gene expression associated with mitochondria seems closely linked with brain connectivity (Fanibunda et al. 2019). Moreover, serotonin and BDNF signaling pathways work synergistically to contribute toward synaptic plasticity and neurogenesis in brain areas (Martinowich and Lu 2008).
4. **Dopamine**
   Biosynthesis: Dopamine is not able to cross the blood-brain barrier and gets synthesized from L-phenylalanine. This essential amino acid is converted to L-tyrosine by phenylalanine hydroxylase in the presence of tetrahydrobiopterin (BH4), iron, vitamin B3, and NADH as cofactors.

L-tyrosine is then converted by tyrosine hydroxylase into 3,4-dihydroxyphenylalanine (L-DOPA). A third reaction by the help of dopamine decarboxylase then converts L-DOPA to dopamine in the presence of vitamin B6 and carbon dioxide (Kuhar et al. 1999).

Role: Dopamine is a catecholamine (catechol ring in their structures) neurotransmitter which has several important functions in learning, memory, and the regulation of mood, motivation, and movement. A number of studies have demonstrated that the endogenous neurogenesis in the adult SVZ is stimulated by dopamine through activation of the dopamine 2 (D2) and dopamine 3 (D3) receptors (Winner et al. 2009).

Considering the significance of neurotransmitters in the neuro-restorative process, it seems crucial to ensure adequate daily intake of amino acids, vitamins, and minerals involved in their biosynthesis for better outcome in patients recovering from ischemic stroke. The mood elevation and sense of well-being due to adequate bioavailability of neurotransmitters would decrease psychological stress in patients leading to lower levels of cortisol. This would protect the patient against the adverse effects of chronic stress, viz., pro-inflammatory signaling cascades, further neuronal damage, and increased risk of stroke recurrence (Table 13.3).

13.3.2 Angiogenesis During Neurogenesis

In human embryos, nervous and vascular systems develop in coordination with each other. Even after a brain injury, neurogenesis and angiogenesis are simultaneously stimulated, which leads to synchronized regeneration of neurons and blood vessels (Tata et al. 2016). Moreover, angiogenesis seems particularly important as multiple studies have reported neuronal migration to be guided by blood vessels (Bovetti et al. 2007; Snapyan et al. 2009; Whitman et al. 2009; Le Magueresse et al. 2012). It is therefore important to understand signaling pathways of angiogenesis alongside neurogenesis (Fig. 13.5).

Role of Wnt Signaling in Angiogenesis

β-Catenin is a crucial modulator of angiogenesis that helps neuronal cells proliferate and survive by inducing the expression of vascular endothelial growth factor (VEGF), one of the central angiogenic factors (Martowicz et al. 2019). The β-catenin signaling is activated when wingless-related integration site (wnt) binds to the seven-pass transmembrane Frizzled (Fz) receptor and low-density lipoprotein receptor-related protein 5/66 (LRP5/6) to form a receptor complex This complex activates the scaffolding protein Dishevelled (Dvl) which further phosphorylates LRP5/6. Phosphorylated LRP5/6 then recruits the axin complex, comprising glycogen synthase kinase 3β (GSK-3β) and casein kinase 1α (CK1α). As a result, axin-mediated phosphorylation/degradation of β-catenin gets disrupted, thereby ensuring the stable availability of β-catenin for the

Table 13.3 List of dietary supplements targeting biomolecules involved in neurotransmitter biosynthesis

Target biomolecule	Compound	Activity	Type of study	References
Tryptophan hydroxylase	Iron	Activation	In vitro	Kuhn and Hasegawa (2020)
L-DOPA	*Mucuna pruriens*	Activation	In vitro	Lampariello et al. (2012)
	Resveratrol	Activation	In vivo (rat)	Khan et al. (2010)
	Gastrodin	Activation	In vivo (mice)	Kumar et al. (2013)
	Baicalein	Activation	In vitro	Li et al. (2004)
GABA	Ajwain seed dry	Activation	In vivo	Latha et al. (2018)
	Actaea racemosa	Activation	In vitro	Cicek et al. (2010)
	Licorice	Activation	In vitro	Cho et al. (2010)
	Epigallocatechin 3-gallate	Activation	In vitro	Campbell et al. (2004)
	Apigenin	Activation	In vitro	Campbell et al. (2004)
Iron	Vitamin C	Activation	In vitro	Lane and Richardson (2014)
Nor-epinephrine	Quercetin	Activation	In vivo (mice)	Choi et al. (2018)
	Vitamin C	Activation	In vitro	May et al. (2012)
Vitamin B6	Bottle gourd	Activation	In vitro	Hasan et al. (2013)
Selenium	Piperine	Activation	In vitro	Bhardwaj et al. (2002)
Copper	*Amaranth* seeds	Activation	In vitro	Bratovcic and Saric (2019)
	Ragi	Activation	In vitro	Rao and Deosthale (1983)
	Gingelly seeds, black	Activation	In vitro	Qadeer et al. (2014)
Serotonin	*Griffonia simplicifolia*	Activation	In vitro	Lemaire and Adosraku (2002)
	St. John's wort	Activation	In vivo (cat)	Fornal et al. (2001)
	a-Lactalbumin	Activation	In vivo (human)	Booij et al. (2006)
	Curcumin	Activation	In vivo (human)	Warner et al. (2017)
	Tryptophan	Activation	In vivo (human)	aan het Rot et al. (2006)
Vitamin B9	Fava beans	Activation	In vitro	Hefni et al. (2015)
	Beets	Activation	In vitro	Rubóczki and Takácsné Hájos (2019)
	Lentil	Activation	In vitro	Paucean et al. (2018)
Aldehyde dehydrogenase	Pear	Activation	In vitro	Srinivasan et al. (2019)
	Thymoquinone	Activation	In vitro	Laskar et al. (2017)

(continued)

Table 13.3 (continued)

Target biomolecule	Compound	Activity	Type of study	References
Phenylalanine hydroxylase	Tetrahydrobiopterin	Activation	In vitro	Flydal et al. (2019)
Glutamate	Tomato	Activation	In vitro	Sorrequieta et al. (2009)
	Watermelon	Activation	In vitro	Bothaina et al. (2019)
	Pomegranate	Activation	In vitro	Rowayshed et al. (2013)
Tyrosine hydroxylase	Rosmarinic acid	Activation	In vivo (mice)	Kondo et al. (2015)
	Ferulic acid	Activation	In vivo (rat)	Asano et al. (2017)

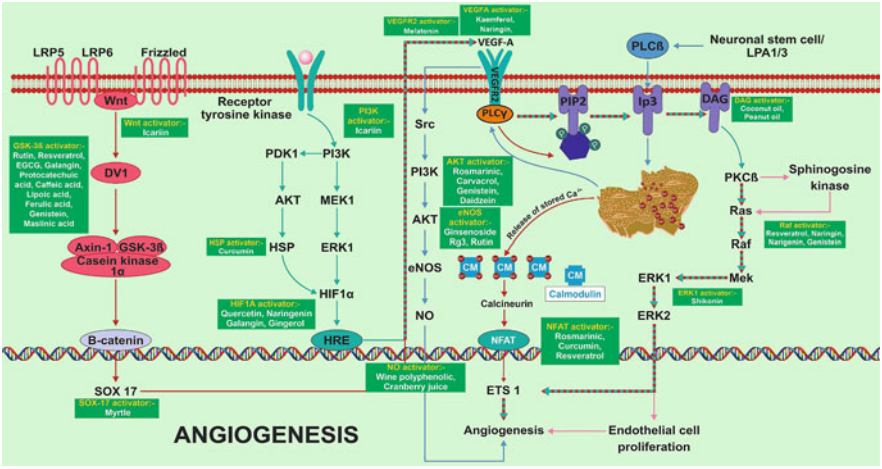

Fig. 13.5 Angiogenesis pathways that work alongside neurogenesis

transcription of SRY-Box Transcription Factor 17 (SOX17) gene (MacDonald et al. 2009).

Role of PLCγ Signaling in Angiogenesis

The SOX17 protein and VEGF-A respectively bind to the VEGF Receptor 2 and activate a common downstream target, i.e., phospholipase C gamma (PLCγ). PLCγ activation can also be triggered by neural stem cells and lysophosphatidic acid. This further carries out the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol triphosphate (IP_3). The IP3 gated channels present on the endoplasmic reticulum releases calcium. Then calcium binds with calmodulin to activate calcineurin, which is a promoter of the transcription factor nuclear factor of activated T cells (NFAT). Moreover, the NFAT switches on the transcription of the ETS-1 gene which is involved in angiogenesis (Olsen et al. 2017; Teo et al. 2009; Liu et al. 2007). Another by-product of PIP2

cleavage is diacylglycerol (DAG). It mediates the activation of Ras-Raf proteins via protein kinase C ß (Kaur et al. 2005; Zachary and Gliki 2001). This is followed by the activation of the MEK-ERK1/2 cascade which is responsible for the transcription of the ETS-1 gene, further causing the upregulation of the vascular endothelial cells (Wang et al. 2020).

Role of Receptor Tyrosine Kinase Signaling in Angiogenesis

The activation of the receptor tyrosine kinase (RTK) upregulates hypoxia-inducible factor 1α (HIF-1α) via the PI3K cascade. PI3K regulates PDK1 and MEK to target HIF-1α via AKT-HSP and ERK1, respectively. Furthermore, HIF-1α translocates to the nucleus and binds with the hypoxia response element (HRE) which finally switches on the transcription of VEGF-A protein (Iwasaka et al. 1996; Chen and Meyrick 2004). VEGF-A binds to its receptor VEGFR2 and signals through an alternative pathway, i.e., Src-PI3K/AKT, to regulate the activity of endothelial nitric oxide synthase (eNOS). This enzyme is responsible for the production of nitric oxide which causes angiogenesis (Karar and Maity 2011).

The targeting of such signaling pathways that cause angiogenesis seems crucial for three reasons. Firstly, proper blood circulation around the region of injury would help ensure a steady supply of nutrients, which serve as the raw material for neurogenesis and biosynthesis of neurotransmitters. Secondly, the newly formed blood vessels are necessary for transporting therapeutic agents such as stem cells, nanomedicine, and even active constituents that target various pathways. Thirdly, the angiogenesis at the site of injury is essential for enabling the blood vessel-guided migration of neurons. Thus, experimental therapeutics for neural regeneration in ischemic stroke patients may yield better results if the active constituents [depicted in green boxes in Fig. 13.5, Table 13.4] that promote these pathways are used for enhancing angiogenesis.

13.3.3 Lifestyle Interventions for Increasing BDNF

Trophic factors including BDNF, vascular endothelial growth factor (VEGF), and nerve growth factor (NGF) play a crucial role in neurogenesis in regions of the adult brain. Nerve growth factor upregulates the production of BDNF via tropomyosin kinase A leading to the stimulation of neurogenesis (Liu et al. 2014a). BDNF is known for its vital roles in neuronal survival, differentiation, and synaptic modulation (Baik et al. 2020). In a study by Taliaz et al., it was found that BDNF knockdown in the adult dentate gyrus region inhibits neurogenesis (Taliaz et al. 2010).

13.3.3.1 Role of Intermittent Fasting in Neuroplasticity

Neuroplasticity refers to the brain's ability to induce both structural and functional changes in neurons. Various studies suggest the involvement of intermittent fasting

Table 13.4 List of dietary supplements targeting markers involved in angiogenesis

Marker	Compound	Activity	Type of study	References
Endothelial nitric oxide synthase	Ginsenoside Rg3	Activation	In vivo (rat)	Wang et al. (2015)
	Rutin	Activation	In vitro	Ugusman et al. (2014)
Nitric oxide	Red wine polyphenolic compounds	Activation	In vivo (rat)	Andriambeloson et al. (1997)
	Cranberry juice	Activation	In vitro	Maher et al. (2000)
PI3K	Icariin	Activation	In vivo (rat)	Cao et al. (2019)
WNT	Icariin	Activation	In vitro	Huang et al. (2017)
SOX 17	Myrtle	Activation	In vitro	Abruzzo et al. (2020)
HSP	Curcumin	Activation	In vitro	Majumder (2018)
Protein kinase B	Rosmarinic	Activation	In vitro	Rong et al. (2018)
	Carvacrol	Activation	In vitro	Fan et al. (2015)
	Genistein	Activation	In vitro	Moran et al. (2014)
	Daidzein	Activation	In vitro	Jin et al. (2017)
HIF-1 alpha	Naringenin	Activation	In vitro	Aditi Sarkar et al. (2012)
	Galangin	Activation	In vitro	Park et al. (2008)
	Gingerol	Activation	In vivo (rat)	Yon et al. (2011)
	Quercetin	Activation	In vitro	Wilson and Poellinger (2002)
ERK1	Shikonin	Activation	In vitro	Chang et al. (2010)
DAG	Coconut oil	Activation	In vivo (mice)	Lu et al. (2020)
	Peanut oil	Activation	In vivo (mice)	Lu et al. (2020)
Rapidly Accelerated Fibrosarcoma-1 (Raf1)	Resveratrol	Activation	In vitro	Yu et al. (2001)
	Naringin	Activation	In vitro	Dong-Il Kim et al. (2008)
	Naringenin	Activation	In vitro	Dong-Il Kim et al. (2008)
	Genistein	Activation	In vitro	Li et al. (2008)

(continued)

Table 13.4 (continued)

Marker	Compound	Activity	Type of study	References
Glycogen synthase kinase-3 beta (GSK-3beta)	Rutin	Activation	In vitro	Wu et al. (2017)
	Resveratrol	Activation	In vivo (mice)	Varamini et al. (2014)
	EGCG	Activation	In vitro	Lin et al. (2009)
	Galangin	Activation	In vitro	Gwak et al. (2011)
	Protocatechuic acid	Activation	In vitro	Huang et al. (2020a)
	Caffeic acid	Activation	In vitro	Lee et al. (2009)
	Lipoic acid	Activation	In vivo (mouse)	Deslauriers et al. (2013)
	Ferulic acid	Activation	In vivo (rat)	Gim et al. (2013)
	Genistein	Activation	In vivo (mice)	El Touny and Banerjee (2007)
	Maslinic acid	Activation	In vivo (mice)	Qian et al. (2015)
Nuclear factor of activated T cells (NFAT)	Rosmarinic	Activation	In vitro	Kang et al. (2003)
	Curcumin	Activation	In vitro	Kliem et al. (2012)
	Resveratrol	Activation	In vitro	Huang et al. (2019)
Vascular endothelial growth factor (VEGF)	Kaempferol	Activation	In vivo (human)	Hu et al. (2020)
	Naringin	Activation	In vivo (rat)	Rong et al. (2012)
VEGFR2	Melatonin	Activation	In vitro	Cerezo et al. (2017)

in neuroplasticity. Brain energy source is glucose, and during metabolic switching, humans retrieve glucose from carbohydrates and fat (during fast). The resulting molecular and cellular adaptations enhance the brain's resistance to injury, disease, and stress (Baik et al. 2020).

Takuya Kishi et al. found that upregulation of BDNF improves cognitive decline in calorie-restricted rat groups (Kishi et al. 2015). Intermittent fasting or calorie restriction causes activation of glutamate neurotransmitters in the brain which binds to its receptors and causes calcium influx. Release of calcium leads to the activation of CAMKII (calcium/calmodulin-dependent protein kinase II) which further leads to the activation of BDNF via CREB. BDNF binds to its receptor tropomyosin receptor kinase B (TrkB) which causes tyrosine phosphorylation of Son of Sevenless (SOS), Src homology and Collagen (SHC), and growth factor receptor-bound protein 2 (GRB2) proteins. These proteins further activate phosphatidylinositol 3-kinase

(PI3K)/AKT pathway and finally lead to neuronal cell proliferation causing neuroplasticity (Rajadhyaksha et al. 1999). Several studies indicate that fasting affects the management of energy metabolism and synaptic plasticity by upregulating BDNF, an essential neurotrophin. Thus, intermittent caloric restriction needs to be explored for enhancing neuro-restorative processes among ischemic stroke patients.

13.3.3.2 Role of Exercise in Neuroplasticity

In a study of Hauer et al., a regimen of functional and progressive resistance training was provided to patients of mild to moderate dementia. It was found that strength and functional training significantly improved motor function like postural control and dynamic balance, sitting and standing ability, and walking, but no significant result was found in improving cognition (Hauer et al. 2012). Liu-Ambrose et al. suggested that 12 months of progressive resistance training improves selective attention, conflict resolution, and quadriceps muscle power (Chen et al. 2016).

(a) Upregulation of BDNF: A combined study of human and animal models by Wang et al. also shows that mild-to-moderate aerobic exercise increased levels of BDNF and FNDC5 (fibronectin type III domain containing 5) while enhancing microglial activation, neuroprotection, cognition, and memory in Alzheimer's disease and associated dementia (Wang and Holsinger 2018; Wrann et al. 2013). Moderate-to-high intensities of aerobic exercise improve the level of antioxidants, eNOS (endothelial nitric oxide synthase), BDNF, and other growth factors and reduce the levels of ROS (reactive oxygen species), neuroinflammation, Aβ plaques, and tau in cognitive regions. As a result of which, cerebral blood flow increases and memory gets enhanced (Chen et al. 2016). It was also found that single bouts of acute exercise for minutes to an hour are beneficial to elevate the level of neurotransmitters like serotonin, dopamine, norepinephrine, acetylcholine, GABA, and glutamate and thus significant for elevating mood, cognition, and memory (Basso and Suzuki 2017).

(b) Upregulation of VEGF: Since neurogenesis and neuronal migration are known to take place alongside angiogenesis, upregulation of VEGF seems important for improved recovery after stroke. In a study by Lei Cao et al., it was seen that inhibition of VEGF expression by RNA interference restricted the phenomenon of neurogenesis (Cao et al. 2004). Heavy exercise induces eNOS production which leads to the stimulation of VEGF. Neuroprotective roles of VEGF have been implicated under stress conditions through the downstream activation of the PI3K/Akt signaling cascade (Wick et al. 2002; Jin et al. 2000).

Considering the role of exercise in increasing BDNF and VEGF, customized exercise regimens for stroke patients may serve the dual purpose of physical rehabilitation and neuroregeneration (Table 13.5, Fig. 13.6).

Table 13.5 List of dietary supplements targeting biomolecules involved in neurogenesis

Target biomolecule	Compound	Activity	Type of study	References
Tropomyosin receptor kinase A (TrkA)	Epigallocatechin-3-gallate	Activation	In vivo (mice)	Liu et al. (2014b)
Nerve growth factor	Daidzein	Activation	In vitro	Min Lin et al. (2010a, b)
Serotonin	Griffonia simplicifolia	Activation	In vitro	Lemaire and Adosraku (2002)
	St. John's wort	Activation	In vivo (cat)	Fornal et al. (2001)
	a-Lactalbumin	Activation	In vivo (human)	Booij et al. (2006)
	Curcumin	Activation	In vivo (human)	Warner et al. (2017)
	Tryptophan	Activation	In vivo (human)	aan het Rot et al. (2006)
	Carbohydrates	Activation	In vivo	Prasad (1998)
L-DOPA	*Mucuna pruriens*	Activation	In vitro	Lampariello et al. (2012)
	Resveratrol	Activation	In vivo (rat)	Khan et al. (2010)
	Gastrodin	Activation	In vivo (mice)	Kumar et al. (2013)
	Baicalein	Activation	In vitro	Li et al. (2004)
cAMP-response element binding protein (CREB)	Luteolin	Activation	In vitro	Lin et al. (2012)
Brain-derived neurotrophic factor	Curcumin	Activation	In vivo (rat)	Zhang et al. (2015)
Endothelial nitric oxide synthase	Ginsenoside Rg3	Activation	In vivo (rat)	Wang et al. (2015)
Protein kinase B	Rosmarinic acid	Activation	In vitro	Rong et al. (2018)
	Carvacrol	Activation	In vitro	Fan et al. (2015)
	Genistein	Activation	In vitro	Moran et al. (2014)
	Daidzein	Activation	In vitro	Jin et al. (2017)
PGC-1alpha	Myricetin	Activation	In vivo (mice)	Jung et al. (2017)
FNDC5	High-fat diet	Activation	In vivo (mice)	Kazeminasab et al. (2018)

13.3.3.3 Other Factors Affecting BDNF Level

Role of Dopamine and Serotonin in the Upregulation of BDNF Dopamine binds to its receptor D5R and activates G protein alpha q which further activates phospholipase C (PLC) protein. Activation of PLC protein induces calcium efflux via calcineurin-CamkII pathway that causes activation of CREB (Perreault et al.

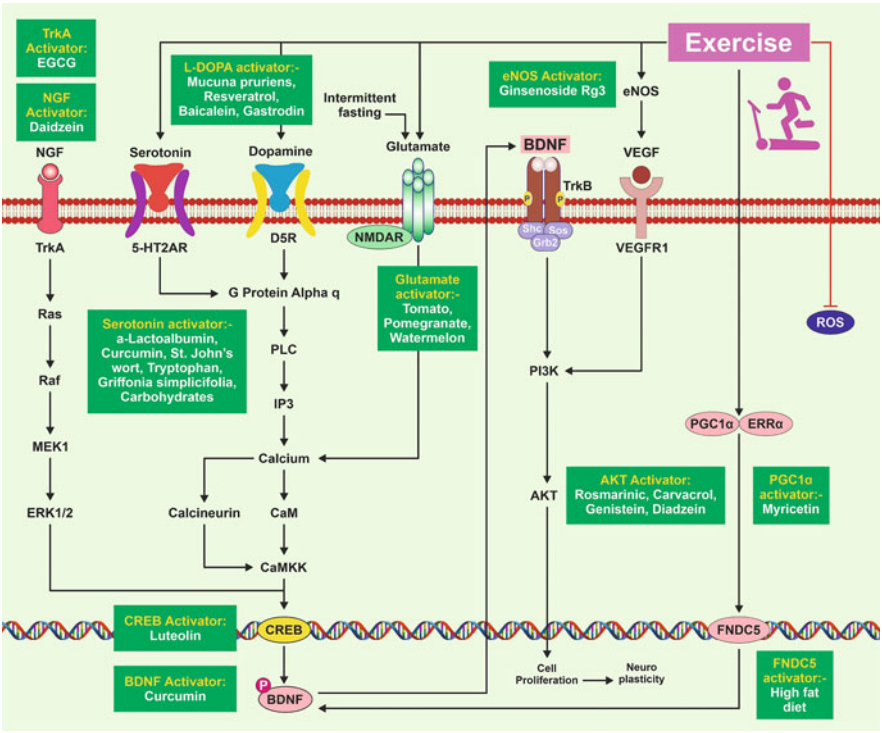

Fig. 13.6 Upregulation of BDNF, NGF, and VEGF for neurogenesis and neuroplasticity

2013). Transcription factor CREB is involved in BDNF-induced gene expression (Xue et al. 2016). Moreover, a study done by Hadoush et al. found dopamine to regulate BDNF in patients with disturbed motor functions (Hadoush et al. 2018).

Similarly, serotonin binds to its receptor serotonin 2A receptor (5-HT2AR) to promote PLC production ultimately leading to the production of BDNF. 5-HT2AR agonists have been reported to regulate BDNF mRNA levels in the hippocampus and neocortex. In vivo study by De-guo Jiang et al. also reported the significance of serotonin in the regulation of BDNF expression during acute psychological stress (Jiang et al. 2016; Vaidya et al. 1997).

Role of Nerve Growth Factor (NGF) Nerve growth factor (NGF) is a neurotrophic factor that regulates proliferation, maintenance, growth, and survival of neurons. NGF binds to tropomyosin receptor kinase A (Trk A) receptor present on neuronal membrane and causes a series of downstream pathways to be activated including Ras-Raf-MAPK-ERK 1/2, leading to the activation of CREB and expression of BDNF (Nguyen et al. 2009).

13.3.4 Role of Interleukins in Neuroregeneration

While many interleukins contribute toward neuroinflammation and recurrence of stroke, some of them trigger cascades that promote synaptogenesis, neurogenesis, and neuroprotection. Understanding these mechanisms would help identify signaling molecules that need to be explored in ischemic stroke for reducing inflammation and improving recovery (Table 13.6, Fig. 13.7).

1. **IL-2-Mediated Neuroprotection**
   IL-2 binds to its receptor IL-2R, composed of the IL-2Rα, IL-2Rβ, and IL-2Rγ subunits. This leads to the activation of Janus kinase 3 (JAK3). Furthermore, PI3K activates the downstream proteins phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Zhao et al. 2006). Thus, phosphoinositide 3 kinase pathway activates the pyruvate dehydrogenase lipoamide kinase isozyme 1 via inositol trisphosphate and ultimately leads to the recruiting of protein kinase B (Kilic et al. 2017). Later on, the mammalian target of rapamycin inhibits protein 53 activity thereby imparting a protective role against neuronal injury. However, it is important to note that IP3 is also linked to the coagulation cascade through downstream signaling (Fig. 13.3).
2. **IL-11-Mediated Astrocytogenesis**
   Astrocytes are involved in neurogenesis and synaptogenesis. IL-11 has been reported to promote astrocytogenesis by activating glial fibrillary acidic protein (GFAP) promoter gene by binding to its receptor glycoprotein 130 (GP130) and further promoting downstream protein dimerization of signal transducer and activator of transcription 3 (STAT3) (Yanagisawa et al. 2000; Ito et al. 2016).
3. **IL-4-Mediated Neurogenesis**
   IL-4 is reported to have a protective role in the brain by promoting neurogenesis. It binds to its receptor IL-4R and causes dimerization of signal transducer and activator of transcription 6 (STAT6) and initiates neural stem cell proliferation (Bhattarai et al. 2016).
4. **IL-3-Mediated Microglial Activation**
   IL-3 secreted by ischemic neurons acts as a pro-inflammatory cytokine. Through its receptor IL-3R, it activates janus kinase 2 (JAK2) and leads to the dimerization of signal transducer and activator of transcription 5 (STAT5). This results in the proliferation and expression of CD40 which leads to microglial activation (Bright et al. 2004).
5. **IL-23, IL-12, and IFN-gamma-Mediated Neuroinflammation**
   IL-23 is involved in the pathogenesis of neuroinflammation. IL-23 is a part of IL-12 cytokine family consisting of heterodimeric cytokines, i.e., p19 subunit and a common p40 subunit shared with IL-12 (Oppmann et al. 2000). The receptor complex of IL-23 consists of two components: IL-12Rβ1 that binds to the common p40 subunit and a specific IL-23 receptor subunit that binds to p19. IL-23 initiates a cellular response by recognizing and binding to its receptor complex and later on activates the cascade JAK/STAT3/4 pathway followed by upregulation and activation of T helper 17 (Th17) cells, which produce various

Table 13.6 List of dietary supplements targeting interleukins and related factors affecting neuroinflammation, neural stem cell proliferation, and glial cell activation

Target biomolecule	Compound	Activity	Type of study	References
STAT3	Butein	Inhibition	In vitro	Trécul et al. (2012)
	Capsaicin	Inhibition	In vitro	Bhutani et al. (2007)
	Ursolic acid	Inhibition	In vitro	Trécul et al. (2012)
	Cryptotanshinone	Inhibition	In vitro	Shin et al. (2009)
	Curcumin	Inhibition	In vitro	Bharti et al. (2003)
	Diosgenin	Inhibition	In vitro	Li et al. (2010)
	Resveratrol	Inhibition	In vitro	Baek et al. (2016)
IL-2	Kaempferol	Activation	In vitro	Asai et al. (2005)
	Daidzein	Activation	In vivo (cow)	Liu et al. (2014c)
	Glucosamine	Activation	In vivo (mice)	Venugopalan et al. (2011)
	Sulforaphane	Activation	In vivo (mice)	Thejass and Kuttan (2006)
	Diosmetin	Activation	In vitro	Ham et al. (2014)
	Apigenin	Activation	In vitro	Granato et al. (2017)
mTOR	Diosmetin	Activation	In vitro	Liu et al. (2016)
	N-Acetyl L-cysteine	Activation	In vivo (mice)	Lin et al. (2020)
Protein 53	Epigallocatechin 3-gallate	Inhibition	In vitro	Zhao et al. (2021)
Protein kinase B	Rosmarinic acid	Activation	In vitro	Rong et al. (2018)
	Carvacrol	Activation	In vitro	Fan et al. (2015)
	Genistein	Activation	In vitro	Moran et al. (2014)
	Daidzein	Activation	In vitro	Jin et al. (2017)
Protein 35	Myricetin	Inhibition	In vitro	Karunakaran et al. (2019)
IL-11	Curcumin	Activation	In vivo (mice)	Toden et al. (2017)
	Resveratrol	Activation	In vivo (human)	Abzevary-Ghahfarokhi et al. (2020)
IL-4	Formononetin	Activation	In vitro	Park et al. (2005)
	Daidzein	Activation	In vitro	Park et al. (2005)
	Equol	Activation	In vitro	Park et al. (2005)
IL-3	Genistein	Activation	In vitro	Rao et al. (1995)
IL-23	Naringin	Inhibition	In vivo (human)	Deenonpoe et al. (2019)
	Epigallocatechin 3-gallate	Inhibition	In vitro	Ichikawa et al. (2004)
	Myricetin	Inhibition	In vitro	Kang et al. (2005)
	Luteolin	Inhibition	In vivo (mice)	Zhou et al. (2020)
	Hesperidin	Inhibition	In vivo (mice)	Li et al. (2019)

(continued)

Table 13.6 (continued)

Target biomolecule	Compound	Activity	Type of study	References
	Apigenin	Inhibition	In vitro	Moore et al. (2017)
	Genistein	Inhibition	In vivo (mice)	Wang et al. (2019)
	Naringenin	Inhibition	In vivo (mice)	Martinez et al. (2015)
	Resveratrol	Inhibition	In vivo (mice)	Khera et al. (2019)
IFN-gamma	Genistein	Inhibition	In vivo (rat)	Wang et al. (2008)
	Apigenin	Inhibition	In vivo (mice)	Rezai-Zadeh et al. (2008)
	Luteolin	Inhibition	In vivo (mice)	Rezai-Zadeh et al. (2008)
IL-17A	Naringin	Inhibition	In vivo (mouse)	Ahmad et al. (2015)
	Rosmarinic acid	Inhibition	In vitro	Yang et al. (2021)
	Chrysin	Inhibition	In vivo (mice)	Meng et al. (2017)
	Curcumin	Inhibition	In vitro	Gouda et al. (2018)
	Kaempferol	Inhibition	In vivo (human)	Lee et al. (2018)
	Naringenin	Inhibition	In vivo (mice)	Wang et al. (2018)
	Hesperidin	Inhibition	In vitro	Fu et al. (2018)
	Apigenin	Inhibition	In vivo (human)	Rahmati et al. (2021)
	Luteolin	Inhibition	In vivo (mice)	Lin et al. (2018)
	Rutin	Inhibition	In vivo (mice)	Liu et al. (2018)
	Taxifolin	Inhibition	In vivo (murine)	Yuan et al. (2020)
	Myricetin	Inhibition	In vivo (mice)	Huang et al. (2020b)
	Resveratrol	Inhibition	In vivo (mice)	Kjar et al. (2015)
	Gingerol	Inhibition	In vivo (mouse)	Sheng et al. (2020)
	Carvacrol	Inhibition	In vivo (mice)	Kianmehr et al. (2016)
	Epigallocatechin 3-gallate	Inhibition	In vitro	Hosokawa et al. (2009)
	B-Glucan	Inhibition	In vivo (mice)	Zhang et al. (2017)
	Fisetin	Inhibition	In vitro	Chamcheu et al. (2019)

(continued)

Table 13.6 (continued)

Target biomolecule	Compound	Activity	Type of study	References
	Oleocanthal	Inhibition	In vivo (human)	Patti et al. (2020)
	Tangeretin	Inhibition	In vivo (mice)	Liu et al. (2017)
IL-17 F	Quercetin	Inhibition	In vivo (mice)	Ma et al. (2020)
	Epigallocatechin 3-gallate	Inhibition	In vivo (mice)	Zhang et al. (2016b)
BACE1	Cinnamic acid	Inhibitor	In vitro	Fang et al. (2019)

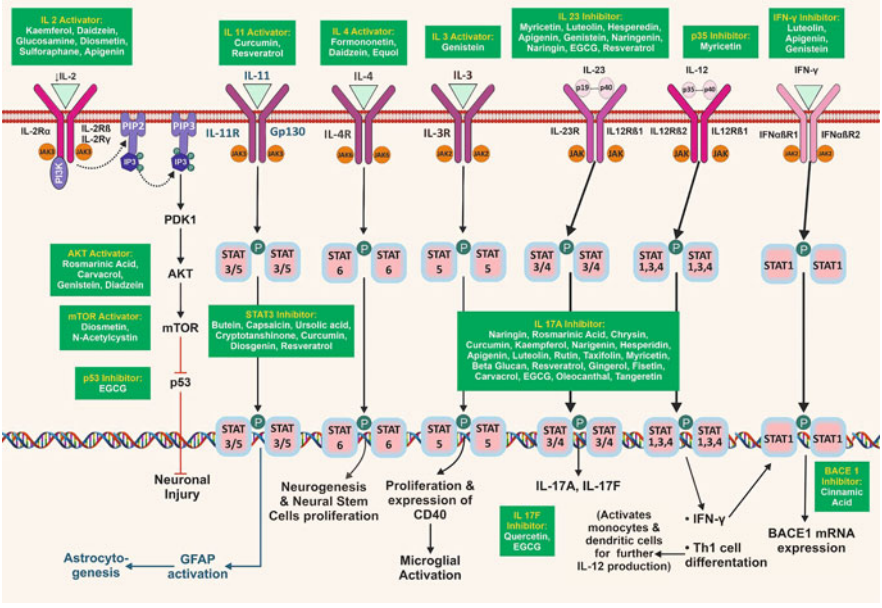

Fig. 13.7 Role of interleukins in neuroregeneration

cytokines including IL-17A and IL-17F which increase inflammation (Sie et al. 2014; Nitsch et al. 2021).

IL-12 is a crucial cytokine which causes the differentiation of T cells into Th1 cells (Abbas et al. 2015). It is a heterodimer composed of p35 and p40 shared with IL-23. IL-12 initiates a cellular response by binding to its receptor, IL-12R, which is composed of two subunits: IL-12Rβ1 and IL-12Rβ2. This further activates the JAK/STAT1/3/4 signaling pathway. The activated STAT proteins upregulates the T helper type 1 (Th1) cells which release IFN-γ leading to neuroinflammation (Finley et al. 2011; Colton 2009). IFN-γ signaling cascade is initiated by

recognizing and binding to its receptor, which is composed of two subunits IFN-α/β and IFN-α/βR2. This activates JAK/STAT1 signaling pathway and the upregulation of the expression of beta-secretase 1 (BACE1) mRNA (Coulson et al. 2010). Beta-secretase 1 is for the proteolytic processing of amyloid precursor protein (APP).

Thus, it is important to further study these signaling cascades in the context of ischemic stroke to identify and target key biomolecules specific to post-ischemic inflammation. Targeted activation and regulation of specific ILs can help optimize the signaling environment for accelerated recovery at the site of injury and prevent further damage due to persistent inflammation.

13.4 Conclusion

The biochemical cascades involved in ischemic stroke and neuronal damage are vastly complex. For better prioritization of research efforts, this chapter highlights some of the key players involved in ischemic stroke and neuroregeneration. There is a need to explore pathways implicated in clotting and inflammation ischemic stroke and test the effectiveness of anti-inflammatory and anticoagulant active constituents in stroke patients. At the same time, experiments that measure the effect of intermittent caloric restriction, customized exercise regimen, and dietary plans for promoting biosynthesis of neurotransmitters need to be conducted in the context of ischemic stroke. Experimental therapies for ischemic stroke would have a greater chance of success if the signaling environment at the site of injury would be conducive for repair mechanisms to take hold.

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