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Ex vivo gene therapy: transplantation of neurotrophic factor-secreting cells for cerebral ischemia

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1. ABSTRACT

Expressions of various neurotrophic factors or their receptors fluctuate after stroke, which in part prompted investigations into the efficacy of neurotrophic factors as treatment modality for stroke. The methods to deliver neurotrophic factors into the brain can be categorized into: 1) the surgical route of administration, such as intracerebral, intraventricular, intra-arterial, or intravenous systemic administration and 2) the manipulation of the therapeutic molecules via *ex vivo* or *in vivo* techniques. With *ex vivo* method, genetically engineered cells, including the use of autologous cells, have been explored. In this review, the potent therapeutic applications of neurotrophic factors in stroke are described, with emphasis on *ex vivo* methods, especially transplantation of encapsulated stem cells modified with adenovirus. Neurotrophic factor delivery, combined with *ex vivo* method, poses as novel treatment for stroke, although additional safety and efficacy studies remain to be examined.

2. INTRODUCTION

Regenerative medicine has been explored in the regenerative organs such as skin, blood (1) and liver (2) or in the ischemia-tolerant organs such as kidney (2) and cornea. Heart and brain are considered as non-regenerative and ischemia-intolerant organs. During the last decade, regenerative medicine for myocardial infarct has been examined. Vascular endothelial growth factor (VEGF) gene transfer using interventional techniques revealed increased myocardial perfusion, suggesting the prospective therapeutic option for myocardial ischemia (3). In addition, endothelial cell progenitors were isolated from human peripheral blood (4) with subsequent transplant study leading to clinical application (5). Arguably, based on recent publications, regenerative medicine for the heart appears more advance than for the brain. Nonetheless, regenerative medicine for the brain has achieved equally important milestones. Neural stem cells with potency for multiple lineage differentiation and self-renewal have been shown to exist in adult mammalian forebrain (6, 7),

indicating that the brain also has the capacity to regenerate. Two major pillars of regenerative medicine using stem cells have emerged, namely exogenous stem cell transplantation and stimulation of endogenous neurogenesis. The source of exogenous neural stem cells consists of embryonic stem cells (8), fetal or adult brains (9, 10), bone marrow (11, 12), umbilical cord (13), or other organs through transdifferentiation or cell fusion (10, 14, 15, 16, 17).

Regenerative medicine for stroke has been recently examined in the laboratory. Transplantation of mesenchymal stromal cell (MSC) from bone marrow to an animal model of stroke revealed the differentiation of MSCs into endothelial cells or neurons (18). Transplantation at 24 hours after middle cerebral artery occlusion (MCAO) demonstrated behavioral improvement, although few MSCs differentiated into neuronal cells suggesting that transplanted cells might have exerted neurorescue effects via secretion of neurotrophic factors. MSC transplantation has also been shown to result in angiogenesis, which appears to precede neurogenesis (19, 20). Thus, stem or progenitor cell transplantation stands as a potent therapy for stroke. Indeed, the neural progenitor cell line human NT2N cells, derived from human teratocarcinoma and fully differentiated into non-dividing neuron-like cells following treatment with retinoic acid and mitotic inhibitors (21, 22), have been transplanted in stroke patients (23, 24). Two to ten million cells were transplanted to patients with a chronic infarct of basal ganglia. Following positron emission tomography with [18F] fluorodeoxyglucose revealed improvement in glucose metabolic activity correlated with motor function (25). Subsequently, histological estimation from one postmortem brain confirmed survival of transplanted cells without tumorigenesis for at least two years in the human brain (26).

Despite scientific advances in our understanding of the disease, the incidence of stroke is increasing worldwide (27). To date, the FDA-approved therapy using recombinant tissue plasminogen activator is only effective to less than 5% of ischemic stroke patients when administered within 3 hours of disease onset (28, 29). Because of this limited therapeutic window, neuroprotection of the stroke brain specifically the penumbra during the acute stage of the disease should be explored for protection and regeneration of injured, but still viable neurons. Neurotrophic factors have been shown to possess both protective and regenerative properties (30, 31).

In contemplating with combined stem cell and neurotrophic factor therapy, some essential points need to be considered: 1) Various phenotypes of neurons, glial cells and endothelial cells are damaged and lose their functions following stroke which differ from the single-neurotransmitter cell depletion pathology seen in neurodegenerative diseases (e.g., dopaminergic neurons in Parkinson's disease or gamma aminobutyric acid [GABA] neurons in Huntington's disease) wherein stem cell and neurotrophic factor treatment have been shown to be effective; 2) Ischemic insult usually occurs suddenly with drastic catastrophic cascade within a short time; 3) Ischemic penumbra is secondary damaged following ischemic core formation, sometimes partly by reperfusion injury. In view of these pathological conditions, an acute therapy for protection or repair of the global brain tissue including pan neural, glial, and endothelial cells, would seem a logical choice for stroke therapy. In this review, we described the utility of neurotrophic factors in stroke, its delivery system with emphasis on *ex vivo* gene therapy, which is the current focus of our laboratory.

3. STRATEGY FOR CEREBRAL ISCHEMIA USING NEUROTROPHIC FACTOR

3.1. Neurotrophic factors and stroke

Expressions of cytokines, neurotrophic factors, their receptors and related molecules involved in signaling pathways vary every second from the onset of ischemic insult to cell death and ultimately the formation of the ischemic lesion. Some exert protective effects on environmental structure in accordance with homeostasis system, although some accelerate cell death. Almost all neurotrophic factors are categorized under the former.

One of the earliest neurotrophic factors implicated in stroke is brain-derived neurotrophic factor (BDNF), which was extracted from porcine brain (32). BDNF was shown to induce neuroprotective effects on ischemic brain via the receptor, TrkB (33). It was also demonstrated that BDNF mRNA was upregulated at 2 hours post-MCAO (34) or at four hours post-photochemical stroke in the hippocampus and cortex of rat brains especially in regions high in N-methyl D-aspartate (NMDA) receptors (35). Recent studies have revealed the mechanisms underlying BDNF neuroprotection. Kiprianova and colleagues demonstrated interaction of BDNF and fibroblast growth factor 2 (FGF2), which is also upregulated after MCAO in stroke (36). FGF2 knock-out mice exacerbated infarct volumes partly due to downregulation of BDNF and TrkB in hippocampal neurons, as well as due to lack of neuroprotective potencies related to FGF2 itself. Chen and colleagues demonstrated that endothelial nitric oxide synthase (eNOS) knock-out mice displayed functional deterioration after MCAO compared to normal mice (37). In their study, eNOS was revealed to increase BDNF expression in the ischemic brain and surprisingly have a strong influence on stem cell proliferation as well as VEGF-induced angiogenic potency. It is interesting to note that FGF2 and eNOS, both bearing angiogenic potency, appear related to neuroprotection by BDNF. Additionally, serial expression of BDNF was observed after bilateral common carotid artery occlusion (BCCAO), a model of moderate ischemia in the rat forebrain (38). Transient BDNF mRNA upregulation at 6 hours post-BCCAO and the return to the control level at day 1 after stroke was seen in the hippocampus, suggesting that the change of cerebral blood flow in the beginning of stroke might affect BDNF gene expression.

In addition to BDNF, VEGF expression recently has been explored extensively in stroke. In a cerebral ischemia model of postnatal rat, expression of hypoxia-

inducible factor 1alpha (HIF1alpha), known as a VEGF-inducer, is increased, following upregulation of VEGF (39, 40). An *in vitro* model of ischemia employing a variety of cells revealed that hypoxia induces flk1 (VEGFR2) and VEGF (41, 42), suggesting VEGF-flk1 binding plays a critical role in mechanisms of biological defense. Many *in vivo* studies using MCAO (43, 44) also support this hypothesis. Undoubtedly VEGF is one of the critical neurotrophic factors in cerebral ischemia.

Furthermore, glial cell line-derived neurotrophic factor (GDNF), which was initially shown to be protective in Parkinson's disease, was also found to promote neuroprotection against cerebral ischemia (45). GDNF and its receptor, GFRalpha1, were upregulated just after cerebral ischemia, suggesting that GDNF triggers neuroprotective effects at very early stages during ischemia (46). Details are described in the accompanying review on GDNF (see Borlongan *et al.*, FBS 2005).

3.2. Protective and regenerative properties of neurotrophic factors in stroke

As described in the *Introduction*, various phenotypes of neurons, glial cells and endothelial cells are damaged altogether immediately after stroke onset. Stem cell therapy may avert these acute pathological conditions. However neuronal differentiation of exogenous neural stem cells and functional reconstruction of neural or glial network have not been fully achieved. In cerebral ischemia, endogenous stem cells are likely activated and could migrate towards the ischemic penumbra, although the number is too small to replace damaged neuronal network (47). Neurotrophic factor might be useful to increase endogenous stem cell to repair damaged neuronal circuit (47). In addition, exogenous stem cell transplantation combined with neurotrophic factor administration or gene therapy for delivery of neurotrophic factors might accomplish an equal or better functional outcome as that accomplished by endogenous stem cell therapy.

For example, BDNF enhances transplanted cell survival and neuronal differentiation. Bone marrow cells from adult rats were transplanted with BDNF ameliorated cell survival and behavioral deficits of MCAO stroke rats, compared to independently administered groups (48). In addition, combination of intravenous administration of BDNF and hypothermia synergistically attenuate glutamate release with subsequent reduction of infarct volumes of rats after MCAO (49), thus revealing direct neuroprotective effects of BDNF. In a similar fashion, Ding and colleagues demonstrated that exercise pre-conditioning by treadmill reduced infarct volumes and ameliorated behavioral score of rats after MCAO by upregulating both BDNF and nerve growth factor (NGF) which coincided with enhanced angiogenesis, suggesting direct and indirect neuroprotective effects upon ischemic insult (50). Furthermore, neuroprotective effects of Cyclosporin A upon hippocampal neurons against ischemic insult have been demonstrated to act on BDNF and TrkB (51). In addition to neuroprotective or neurorescue effects on cerebral ischemia, BDNF modulates neurogenesis in stroke. Larsson and colleagues revealed that intraventricular BDNF injection stimulated neurogenesis in intact brain (52). However, intraventricular injection of adeno-associated virus (AAV) encoded BDNF gene following intrahippocampal transduction surprisingly suppressed neurogenesis in rat brains with global ischemia, suggesting the possibility that administration of certain neurotrophic factors attenuates intrinsic neurogenesis (52). Furthermore they confirmed that endogenous BDNF counteracts neuronal differentiation, but not cell proliferation of ischemic hippocampus using intraventricular injection of TrkB-Fc as BDNF scavenger (53). The group also demonstrated anterograde delivery of BDNF to the ischemic striatum which did not promote neuroprotection, but induced neurogenesis (54). Indeed, a recent study showed that vascular adventitia in the subgranular zone of the hippocampus, abundant in BDNF, generates neuronal progenitor cells in the ischemic non-human primate brains, suggesting the potency of BDNF to enhance neurogenesis (55). These series of study provide various neuroprotective aspects of BDNF.

VEGF also has been demonstrated to exert therapeutic effects in cerebral ischemia. Exogenous VEGF administration into cultured rat hippocampal neurons (56, 57) and cortical neurons (58) induced neuroprotective effects against hypoxic injury via flk1, although upregulated endogenous VEGF proved to be more effective than exogenous VEGF (59). Nonetheless, intraventricular infusion of low dose VEGF protected against cerebral ischemia (60). The low dose and continuous administration of VEGF appear important parameters for angiogenesis with mature vessels (61). In addition, VEGF itself exerts direct neuroprotective and neurorestorative effects on various neurons via flk1 without vascular and astroglial involvement (62, 63). Taken together, these studies indicate that VEGF displays multiple potencies to various cells in the central nervous system, including neurons, endothelial and glial cells, suggesting that VEGF is an efficacious therapeutic agent candidate for brain disorders characterized by death of various cell types, such as the pathology seen in stroke (64). VEGF was also reported to be associated with neurogenesis in murine cortical cultures and in adult rat subventricular zone (SVZ) and hippocampus via flk1 (65). Intraventricular low dose of VEGF injection resulted in a survival promoting effect of VEGF on neural progenitor cells by anti-apoptotic effects via flk1 (66). These investigations further verified neurogenesis induced by VEGF in a cerebral ischemia model (67).

The concept of a "vascular niche" for neurogenesis was advocated by Palmer and colleagues (68). A majority of the precursors in the subgranular zone in hippocampus proliferated in a novel neuroangiogenic focus where neuronal, glial, and endothelial precursors divide in tight clusters, and are commonly found at a branch or terminus of the capillaries, considered as active sites of angiogenesis. The cue for neuroangiogenesis is dependent on humoral factors such as circulating VEGF or signals from the central nervous system (CNS) (68). Shen and colleagues demonstrated that endothelial cells played a critical role in neural stem cell proliferation using co-

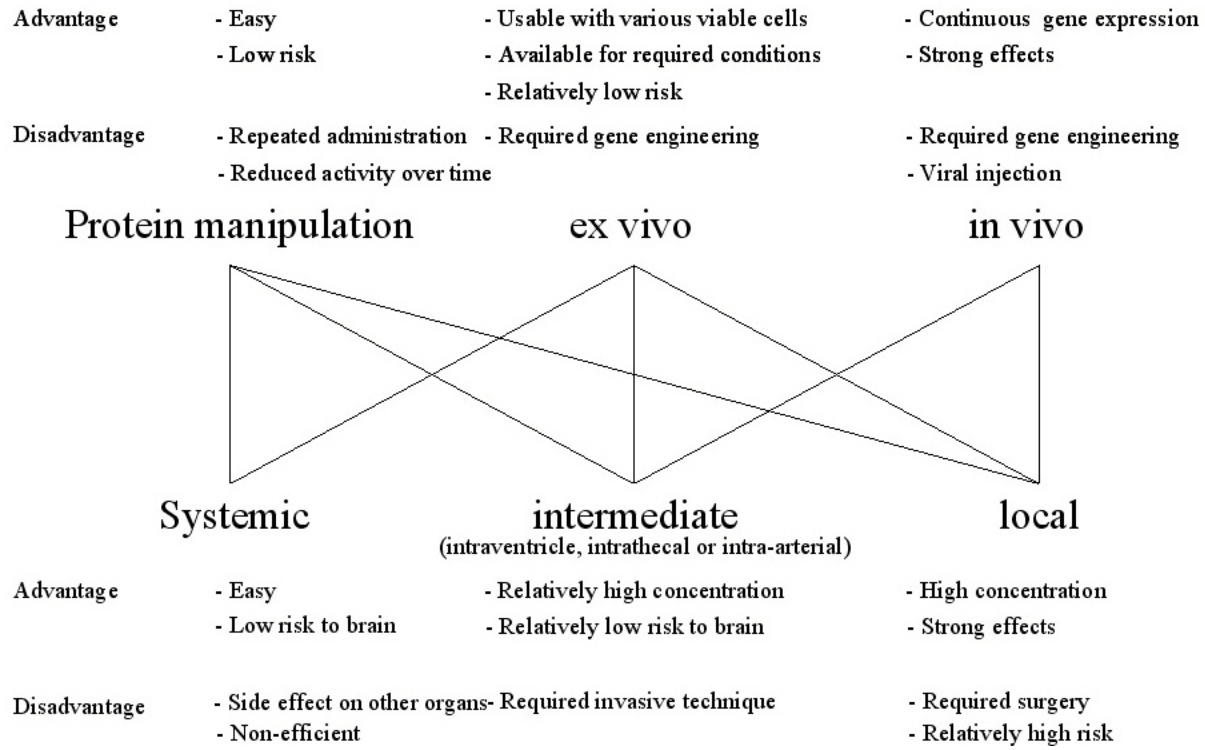

Figure 1. Advantages and disadvantages of neurotrophic factor delivery routes and methods.Surgical route and manipulation method are combined variously. The delivery way should be determined in consideration of each advantages and disadvantages.

culture of endothelial cells and neural stem cells, suggesting involvement of VEGF in neurogenesis (69). Hepatocyte growth factor (HGF) is another angiogenic factor which attenuates ischemic injury by appropriate angiogenesis and anti-apoptotic effects similar to VEGF. A clear distinct advantage of HGF over other neurotrophic factors is that it works without blood brain barrier (BBB) disruption and following brain edema. Details are discussed in the accompanying review (70).

Laboratory studies using cell transplantation support the potency of neurotrophic factors in stroke. Carotid body transplantation to MCAO stroke rats ameliorated behavioral scores and reduced infarct volumes (71), possibly by elevating GDNF, NGF and BDNF level of ischemic penumbra, suggesting that trophic effects of cell therapy might play an important role in stroke therapy (71). Similarly transplantation of GDNF-secreting cells, such as kidney cells and pineal gland, also promoted neuroprotective effects upon cerebral ischemia (72, 73), which parallel therapeutic effects produced by exogenous GDNF application (45, 46).

Apart from neurotrophic factor, erythropoietin, a well-known hematopoietic hormone, was also shown to rescue neuronal cell death after cerebral ischemia (74, 75). Pretreatment of intraventricular erythropoietin injection successfully rescued CA1 hippocampal neurons mainly from nitric oxide (NO)-induced cell death. Furthermore, intravenous administration of erythropoietin was demonstrated to ameliorate clinical outcome of stroke patients without safety concerns (76). Moreover, granulocyte colony stimulating factor (G-CSF) was also revealed to have neuroprotective effects on a stroke model of rat (77, 78). In addition to the neuroprotective effects, G-CSF enhanced exogenous transplanted cell survival or endogenous neurogenesis, similar to that seen with neurotrophic factor treatment (79, 80). Furthermore, some traditional drugs, like minocycline, have been shown to promote neuroprotective effects against stroke (81). Low dose administration of minocycline has been recently shown to exert direct neuroprotective effects via anti-apoptotic pathway, as well as indirect mechanisms related to the drug's anti-inflammatory activity (82). In addition, administration of Atorvastatin, an established lipid-lowering agent, increased VEGF, flk1 and BDNF expression in the ischemic penumbra after stroke in mice with subsequent improvement in functional recovery (83). Thus, many humoral factors including neurotrophic factors, hormones, cytokines, or chemokines might have neuroprotective or neurorestorative potencies on ischemic brains.

3.3. Neurotrophic factor delivery

3.3.1. Surgical route of neurotrophic factor delivery

The different approaches in administering neurotrophic factors can be divided broadly into two categories; 1) the surgical route of delivery, such as local and systemic administration; and 2) the manipulation method, namely, protein manipulation, *ex vivo* and *in vivo* gene techniques (Figure 1).

For surgical route of neurotrophic factor delivery, several laboratory studies abound on Parkinson's disease

research, which can be extrapolated to stroke therapy. Intraputaminal GDNF infusion for Parkinson's disease has been a major subject of interest about neurotrophic factor. Indeed, direct GDNF infusion with catheter was shown effective for functional recovery (84, 85). Historically, all study utilizing intraventricular GDNF administration for Parkinson's disease patients ended up with failure (86, 87). These disappointing results might be due to the intraventricular administration itself which could have prevented GDNF from reaching the target dopaminergic cells of nigrostriatal system. As discussed above, the neuroprotection induced by neurotrophic factors is largely mediated by its receptor, thus the therapeutic molecule's availability in the brain may be required in order to activate downstream pathway for neuroprotection or neuroregeneration. Based on this assumption, intracerebral delivery of neurotrophic factors to discrete brain regions may be optimal for neuroprotection. However, for stroke therapy, local administration of neurotrophic factors to the brain in the acute stage of stroke might include some risks of hemorrhage or non-conducive nutritious environment in the case of cell transplantation which may subsequently lead to poor survival of transplanted cells. On the contrary, systemic administration requires a large volume of the neurotrophic factors which would not be practical and may even produce systemic side effects. Notwithstanding, optimal drug formulation may allow systemic route of delivery as effective. Indeed, intravenous administration of low molecular weight heparin for ischemic patients within 48 hours of stroke onset successfully demonstrated the neuroprotective capacity through prevention of glutamate-induced calcium release (88). If the neurotrophic factor can effectively act in ischemic penumbra through BBB without side effect, intravenous systemic administration will be the most feasible way in the future. Alternatively, intrathecal delivery may also provide optimal benefits with minimal side effects, in that Cao and colleagues demonstrated that bcl-2 gene delivery using cationic liposome method with intrathecal approach ameliorated infarct volumes safely (89).

3.3.2. Protein manipulation, and *in vivo* and *ex vivo* gene manipulation of neurotrophic factors

In this section, protein manipulation, *in vivo* and *ex vivo* gene methods of manipulating neurotrophic factors are described. Direct administration indicates delivery of trophic factor itself, the simplest and basic way of drug treatment with lower risks than other methods using gene engineering. Various factors have been explored with direct administration at first. For example, in order to confirm GDNF administration to the target area, GDNF protein was directly injected to ischemic cortex after squamosal bone removal to MCAO model of rats (90). The treatment reduced infarct volumes accompanied with reduction of NO release from ischemic cortex. Although this study demonstrated strong potency of GDNF for stroke therapy, clinical applications may be limited due to short period of GDNF protein activity, even with high dose, after administration. Recently, a new drug delivery system was developed focusing on protein manipulation. The primary objective of such delivery systems is to facilitate drug entry into and maintain their bioavailability in the brain, with minimally invasive procedures. One such delivery method is the use of TAT peptide, which is composed of 11-amino acid, and easily can cross the cell membrane and BBB. Intravenous administration of TAT-GDNF just after transient MCAO ameliorated ischemic damage by anti-apoptotic effect of GDNF (46, 91, 92). Cell-penetrating peptides, such as TAT or polyarginine, are strong candidates for facilitating neurotrophic factor entry across the BBB, although there are still difficulties in delivery of biologically active protein especially in case of macro molecule proteins (93). In addition, placement of catheter for repeated injections is likely to be required because of reduced activity with very short half-life of some neurotrophic factors.

In vivo method is the direct gene delivery approach to living body including direct injection of viral vector encoding target gene. With such method of neurotrophic factor treatment, strong efficacy is expected despite risks of viral infection. There are many studies using *in vivo* method with lentivirus (94), adeno-associated virus (95) or Herpes simplex virus (96) for treating neurodegenerative diseases including Parkinson's disease. Studies about *in vivo* gene transfer for stroke therapy have been also examined (97, 98). Recently improved viral vectors have been explored, including hemagglutinating virus of Japan (HVJ)-liposome method, known as a macro molecule of gene transfer method (99). Injection of HGF gene incorporated in the HVJ-liposome into the subarachnoid space following parenchymal transfection delayed neuronal cell death in hippocampal CA1 region of gerbils through anti-apoptotic effect including downregulation of Bax (99). In addition, intraparenchymal injection of GDNF gene with herpes simplex virus amplicon, known to express the encoded gene for a short duration, at 4 days before transient MCAO also revealed neuroprotective effect, although injection at 3 days post-MCAO was not effective (100). Further advances in gene engineering might expand the possibility of *in vivo* gene therapy for stroke.

Ex vivo method, on the other hand, involves transferring the gene of interest into cultured cells, then subsequently transplanting these transfected cells. Compared with *in vivo* gene therapy, the *ex vivo* approach poses with relative low risks to host brain cells. In *section 3.4*, *ex vivo* gene therapy is discussed in detail. In both gene delivery methods, advantages and disadvantages of each approach need to be carefully considered and confirmation of the safety is prerequisite to proceeding with clinical application.

3.3.3. Timing of neurotrophic factor delivery

The timing of neurotrophic factor administration is also critical for stroke therapy (Figure 2). The ischemic penumbra is a good target for protective and reparative therapies. However, the following issues should be addressed: 1) The target penumbra area can easily succumb to bleeding especially during reperfusion or anti-coagulant therapy commonly used for stroke. Accordingly, subsequent direct intraparenchymal injection into the penumbra may prove risky and cause a catastrophic

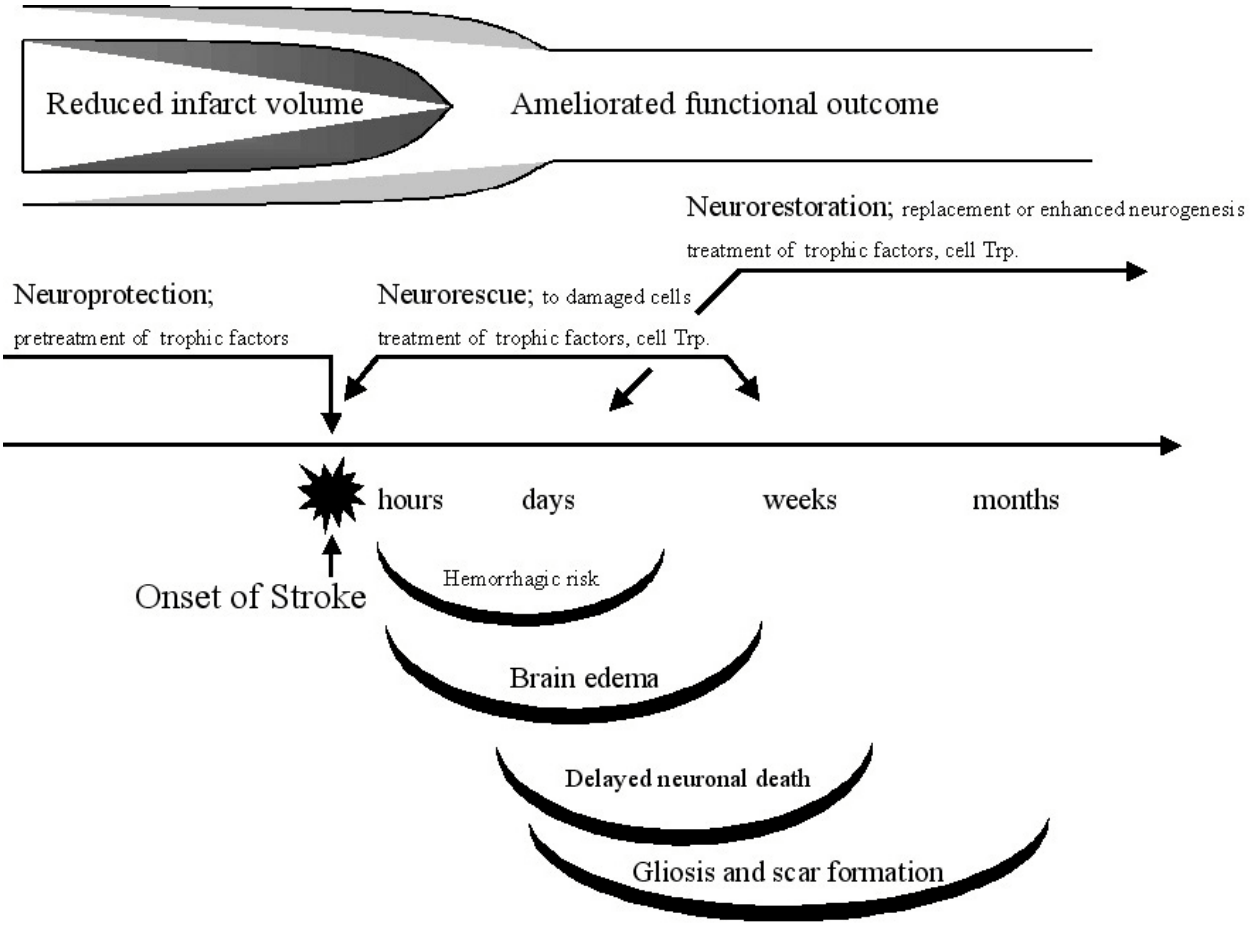

Figure 2. Time table after an ischemic insult and regenerative medicine.After the onset of stroke, microenvironment of the brain varies vertiginously. Treatment might be performed in mind for hemorrhagic risk, brain edema, delayed neuronal death and gliosis. In contrast, neuroprotective and initial neurorescue treatment can reduce infarct volume with subsequent functional recovery. However, delayed neurorescue and neurorestorative treatment might ameliorate functional outcomes without reduced infarct volume.

hemorrhage. 2) After occlusion of vessels, hypoxia can induce cytotoxic edema coupled with BBB disruption due to endothelial hypoxia. The occurrence of severe vasogenic edema may worsen the microenvironment within and around the penumbra (101). Although the BBB disruption enhances bioavailability of trophic factors in the brain, ischemic cells, as well as endogenous stem cells migrating into the penumbra must survive under conditions of increased intracranial pressure. 3) Malnutrition of the ischemic tissue due to poor blood circulation can exacerbate cell death in ischemic penumbra. 4) Increased inflammatory reaction including production of free radicals can accelerate the transformation of the ischemic penumbra to becoming a part of the ischemic core. 5) The formation of the penumbra proceeds over an acute period after stroke onset, which would require immediate intervention within such limited therapeutic window.

The therapeutic time window for neurotrophic factor delivery appears to mimic the narrow treatment period of tPA. Intravenous administration of BDNF at 30 minutes after MCAO, reduced infarct volumes of rat brains by anti-apoptotic effects via Bax downregulation and Bcl-2 upregulation (102). Intravenous BDNF administration at 1 hour post photothrombotic ischemia did not reduce infarct volumes of rat brains, but achieved functional motor recovery and a slight neuronal remodeling (103). On the contrary, BBB targeting system might expand therapeutic time window for stroke. BDNF conjugated to the BBB drug targeting system administered at 2 hours after MCAO reduced cortical infarct volumes of rat brains, but not subcortical infarct. Similarly, delayed administration of BDNF conjugated to the anti-transferrin receptor antibody at 2 hours after MCAO also displayed neurorescue effects on the ischemic rat brain (104, 105).

Cell therapy also revealed the importance of the stroke therapeutic time window. Intravenous administration of green fluorescent protein (GFP)-labeled human umbilical cord blood cells during MCAO with mannitol as BBB permeabilizer demonstrated functional recovery and reduced infarct volumes of rat brains, accompanied with

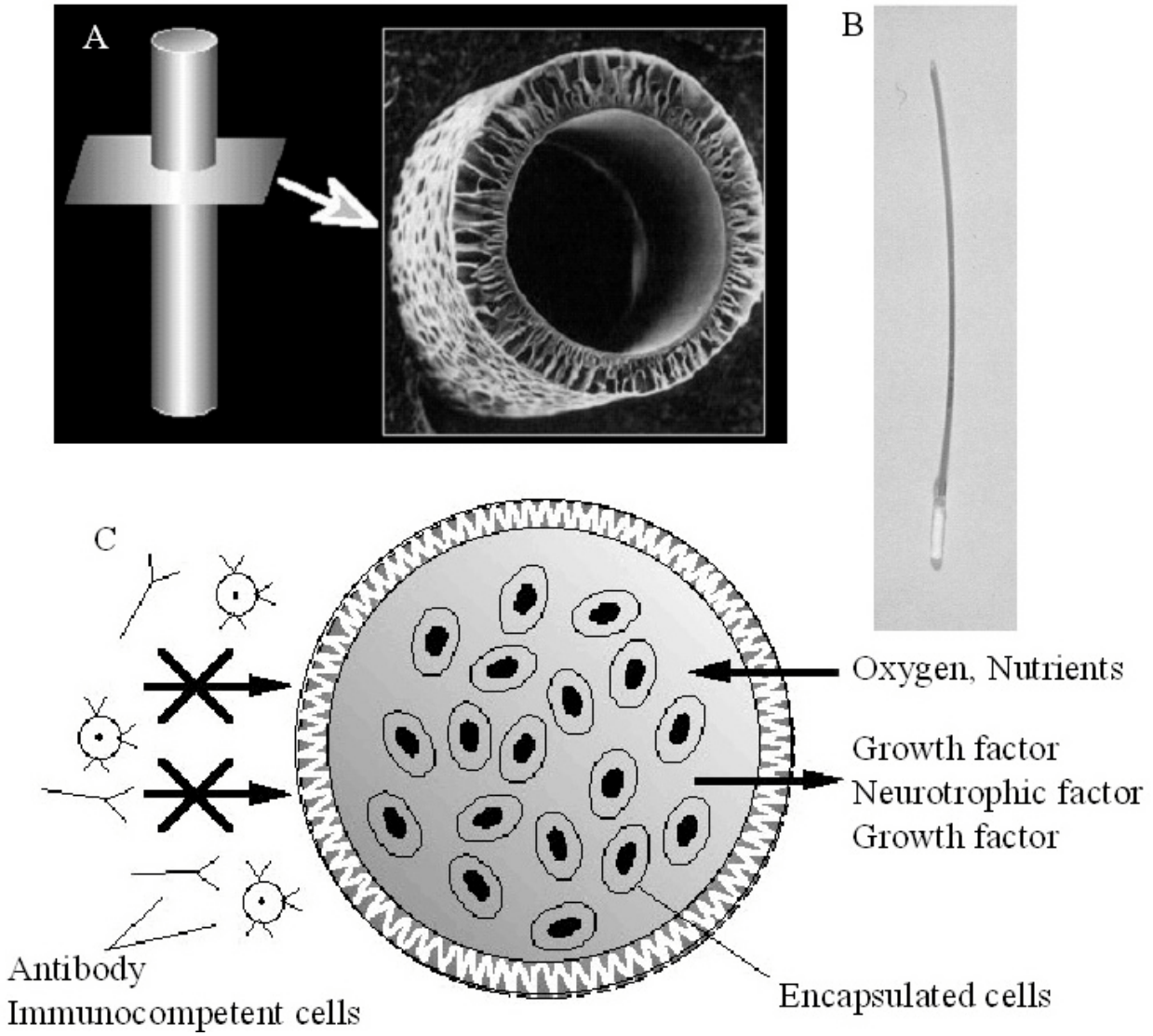

Figure 3. Scheme of encapsulated cells. Capsules are made with semipermeable membrane. Oxygen and nutrition can freely move into the capsule, although antibodies and immunocompetent cells can't pass through the membrane. In addition, the trophic factor or neurotransmitter secreted from cells inside the capsule can be expanded around the capsule (A). Transplantation of capsules with tether can be easily retrieved outside brain whenever needed (B, C). Microscopic view of the capsule demonstrated porous structure of the membrane (D).

increased GDNF levels in the brain (106). However no GFP-labeled cells were detected in the ischemic penumbra immunohistologically, suggesting that GDNF secreted from transplanted cells passed through BBB due to mannitol and displayed neurorescue effects at the very acute stage of infarct without replacement with damaged cells (106). Li and colleagues also demonstrated that transplanted cells worked as a supplier of neurotrophic factors (107). Intravenous administration of human MSC at 1 day post-MCAO revealed upregulation of BDNF and NGF and decreased number of apoptotic cells in the ischemic penumbra with functional recovery. Furthermore, the endogenous neurogenesis was enhanced in the SVZ in the transplanted group (107). In addition, BDNF transduced human adipose tissue-derived stromal cells, obtained from liposuction tissues and induced to differentiate into neurons with azacytidine, were administered to rat brains at 1 day after MCAO. Transplanted cells survived and migrated towards the site of injury, which accompanied functional recovery, but infarct volumes were not reduced (108). Embryonic stem cells transduced *v-myc* oncogene and used in the similar study demonstrated ameliorated behavioral scores with reduced atrophy at 56 days after transplantation but not at 7 days (109). Based on the inconsistencies in ameliorations of behavioral and histological deficits produced by stroke following different dosing regimens, additional studies are warranted to reveal the most appropriate timing of administration of neurotrophic factors or cell transplantation.

3.4. *Ex vivo* gene therapy

Ex vivo gene therapy has specific advantages compared to other delivery systems (110), including the following: 1) Promising cells with appropriate secretion of specific factors can be selected before transplantation. 2) Viral transfection to cells was performed in the laboratory, thus reducing the risks of using live replication-competent virus transfer to the patients directly. 3) Viable and appropriate cells are usable by tailor-made engineering instead of targeting degenerating host cells which likely display low viability. With these advantages in mind, we described here two different *ex vivo* methods generated in our laboratory.

3.4.1. Encapsulated cell transplantation

Our group has pursued the investigation of encapsulated cell transplantation for treating CNS diseases, including Parkinson's disease and cerebral ischemia (111, 112, 113, 114,115, 116, 117). Encapsulated cell transplantation has the following characteristics (Figure 3). 1) Various neurotransmitters, neurotrophic factors, or growth factors can be produced continuously from encapsulated cells with engineered properties. Cells inside the capsule are supplied sufficient nutrient and oxygen through the semipermeable membrane. 2) Scant immune reactions and no immunological rejection arise because cells inside are protected by stiff envelope. In addition, there is no risk of tumor formation in the host tissue. 3) The capsule can be removed from the transplanted brain if need be. 4) Various cells including immortalized cell lines can be transplanted safely as a surviving donor with no ethical problems. These cells are also engineered genetically with ease. Thus, encapsulated cell transplantation is a suitable method of neurotrophic delivery using *ex vivo* gene therapy.

Our group has reported encapsulated cell transplantation for MCAO model of rats. FGF2 secreting cell line (baby hamster kidney cell (BHK)-FGF2) was made using a cationic liposome-mediated DNA delivery system. One million cells were encapsulated, subsequently secreted about 20ng per day for at least 6 months. Permanent MCAO was performed using an intraluminal suture technique at 6 days post-transplantation of the capsule into the striatum of rats. The number of terminal deoxynucleotidyl transferase-mediated biotynilated UTP nick end labeling (TUNEL) positive cells at 12 hours and infarct volumes at 24 hours post-MCAO significantly reduced and angiogenesis at 6 days post-MCAO increased in the BHK-FGF2 group compared to the control group. This study revealed neuroprotective effects of FGF2 upon a MCAO model of rat by anti-apoptotic mechanisms. Other factors like VEGF (118) or GDNF (46, 90) also have neuroprotective effects upon a MCAO model of rat with subsequent behavioral improvement and reduced infarct volumes. The direct anti-apoptotic mechanism via each receptor, e.g., flk1 to VEGF or GFRalpha1 to GDNF, as well as the indirect mechanisms through angiogenesis and glial proliferation (116, 117) possibly mediated this VEGF/GDNF neuroprotection. In addition, encapsulated cell transplantation enabled us to deliver continuous and low dose administration of secreting factors. Low dose administration of VEGF using encapsulation technique achieved highly neuroprotective effects on dopaminergic neurons, although high dose induced severe brain edema with subsequent decreased neuroprotective effects (119). Other groups also reported that intraventricular administration of low dose of VEGF exerted neurorescue effects on stroke model of mice, but not intravenous administration of high dose VEGF (120).

Transplantation of neurotrophic factor-secreting cells has been shown effective also against stroke. Transplantation of encapsulated choroid plexus from adult rats or young porcine reduced infarct volumes of rats after MCAO and ameliorated behavioral deficits. Parallel *in vitro* studies demonstrate the neuroprotective effects of conditioned media harvested from cultured choroid plexus, further implicating the secretion of neurotrophic factors from the encapsulated choroid plexus (121, 122).

Neuroprotective effects of GDNF have also been demonstrated in neonatal cerebral ischemia using a modified Levine's model (123, 124). Neonatal rats received hypoxic-ischemic stress at 2 days post-transplantation of GDNF secreting encapsulated cells to 7 or 12 day-old-rats. Histological brain damage as well as learning and memory impairment reduced in the treatment group, indicating neuroprotective potencies of GDNF to ischemic insult to neonatal brains. Neurorestorative effects of GDNF are now explored to approximate clinical condition. These studies might shed light on the GDNF potency for severe asphyxia of newborn in the future.

Transplantation of encapsulated cells holds great promise for clinical applications in various CNS diseases (125). In addition, encapsulation technique might contribute to the development of neural stem cell therapy. There is little evidence of cell replacement of damaged cells, as well as re-innervation of the host CNS with neural stem cell transplantation. Moreover, the ratio of neuronal differentiation of stem cells derived from non-neuronal source is very limited (109). Despite the minimal neuronal differentiation and host re-innervation, the occurrence of functional recovery and reduced infarct volumes in transplanted stroke animals suggest that expression of humoral factors by stem cells is a more plausible explanation for such robust neuroprotection (126). Neural stem cells are also reported to secrete neurotrophic factors, including pleiotrophin and GDNF, suggesting that these factors promote differentiation to dopaminergic neurons and extensive host axonal growth after spinal cord injury (109, 127). Neurotrophic factors can be secreted from the capsule, although there is no re-innervation of the host achieved with encapsulated cell transplantation. A thorough comparison of neural stem cells with and without encapsulation might shed crucial light on the specific biology of transplanted neural stem cells. Furthermore, selective differentiation of neural progenitor cell has been achieved by high-epitope density nanofibers (128), thus suggesting the potential of using the capsule as a scafford for neuronal differentiation.

3.4.2. Neurotrophic factor-secreting stem cell transplantation

Recently our group examined *ex vivo* gene therapy using various stem cells. This method achieves

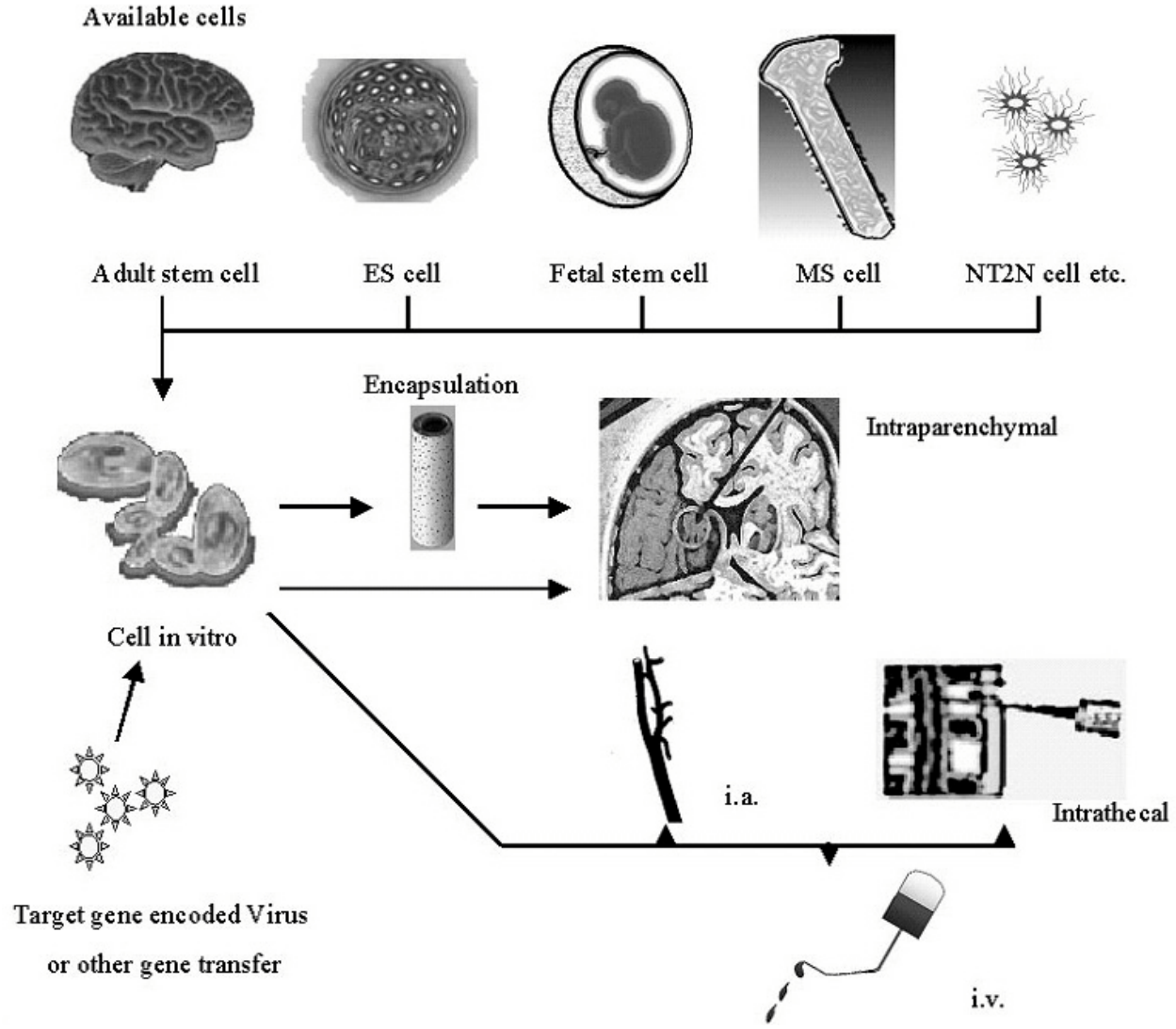

Figure 4. Concept of *ex vivo* gene therapy.Various types of cells are available for *ex vivo* gene therapy. Cells are isolated and cultured then processed for gene transfer. If need be, cells can be differentiated. After confirming potencies to secrete target factor, cells are transplanted with or without encapsulation by intraparencymal injection, intraventricular, intrathecal, intra-arterial, or intravenous administration.

wide spread distribution of trophic factors owing to the migration ability of the cells. MSC from bone marrow has been reported to have neuroprotective and neurorestorative effects in a rat model of stroke as described above (11). A clinical trial of intravenous administration of autologous bone marrow cells to patients of cerebral infarcts was recently initiated in Japan based on their preclinical studies (129, 130). The group has another plan for embarking on autologous MSC transplantation using cryopreserved MSC, harvested and isolated in advance from high risk patients. These trials might lead to a breakthrough for stroke therapy, although the severity and location of infarcts or the timing of cell transplantation are critical factors that remain to be optimized.

Transplantation of human MSCs genetically modified to secrete GDNF and BDNF has been shown effective in MCAO stroke animals (131, 132). BDNF secreting cell line was established using a fiber-mutant F/RGD adenovirus vector (133, 134) and transplanted at 24 hours after transient MCAO. Behavioral tests revealed functional recovery in the treatment group at 7 and 14 days post-transplantation, with corresponding reduction of infarct volumes on magnetic resonance imaging analyses. These therapeutic effects might be due to anti-apoptotic effects of BDNF via TrkB (135). Furthermore, only a few transplanted cells differentiated to mature neurons or astrocytes, supporting the hypothesis that secreted BDNF played an important role in the therapeutic effects in this study. These studies indicate that transplantation of trophic

factor-secreting MSCs possesses wide clinical applications for stroke therapy.

Neural stem cells are also likely candidate for transplantation therapy in stroke, as they also secrete several neurotrophic factors secreted from transplanted cells. In order to enhance the donor cells' capacity to secrete neurotrophic factors, adult neural stem cells from SVZ were genetically engineered via neurosphere amplification technique with epidermal growth factor and finally transfected with adenovirus containing GDNF gene. These genetically modified neural stem cells exhibited almost the same potency to differentiate into neurons and astrocytes. Transplantation of the engineered cells before or after MCAO resulted in behavioral improvement and reduction of the infarct volumes. These observations support clinical application of GDNF-secreting stem cells in stroke. Furthermore, autologous neural stem cells with or without gene transfer are equally efficacious alternative graft source (136). Thus, *ex vivo* gene therapy stands as a feasible therapeutic option for stroke patients (Figure 4).

4. PERSPECTIVE

Studies on trophic factors or growth factors for stroke have increased exponentially in recent years. The appropriate route, method and timing need to be optimized in order to advance neurotrophic factor therapy the clinic. A multidirectional strategy using recent technologies including cell engineering and *ex vivo* gene therapy will facilitate optimization of neurotrophic factor treatment. Ongoing clinical trials of cell transplantation, as well as administration of neurotrophic factors will need to be critically evaluated. Rigid criteria for the safe conduct and effectiveness of newly established therapies should be in place before regenerative medicine truly becomes one of routine treatment options in our field.

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Abbreviations: BBB: blood brain barrier, BCCAO: bilateral common carotid artery occlusion, BDNF: brain-derived neurotrophic factor, BHK cell: baby hamster kidney cell, CNS: central nervous system,

eNOS: endothelial nitric oxide synthase, FGF2: fibroblast growth factor 2, GABA: gamma-aminobutyric acid, G-CSF: granulocyte colony stimulating factor, GDNF: glial cell-line neurotrophic factor, GFP: green fluorescent protein, HGF: hepatocyte growth factor, MCAO: middle cerebral artery occlusion, MSC: mesenchymal stromal cell, NGF: nerve growth factor, NMDA: N-methyl D-aspartate, NO: nitric oxide, SVZ: subventricular zone, TUNEL: terminal deoxynucleotidyl transferase-mediated biotynilated UTP nick end labeling, VEGF: vascular endothelial growth factor

Key Words: Nervous system, Brain, Treatment, Therapeutics, BDNF, encapsulation, *ex vivo*, GDNF, gene therapy, neurotrophic factor, stem cell, stroke, Cytokine, VEGF, Review

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