Biotherapies in neurological diseases

Biotherapies in stroke

Biothérapies dans l’accident vasculaire cérébral

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Abstract

Stroke is the second leading cause of death worldwide and the most common cause of severe disability. Neuroprotection and repair mechanisms supporting endogenous brain plasticity are often insufficient to allow complete recovery. While numerous neuroprotective drugs trials have failed to demonstrate benefits for patients, they have provided interesting translational research lessons related to neurorestorative therapy mechanisms in stroke. Stroke damage is not limited to neurons but involve all brain cell type including the extracellular matrix in a “glio-neurovascular niche”. Targeting a range of host brain cells, biotherapies such as growth factors and therapeutic cells, currently hold great promise as a regenerative medical strategy for stroke. These techniques can promote both neuroprotection and delayed neural repair through neuro-synaptogenesis, angiogenesis, oligodendrogliogenesis, axonal sprouting and immunomodulatory effects. Their complex mechanisms of action are interdependent and vary according to the particular growth factor or grafted cell type. For example, while “peripheral” stem or stromal cells can provide paracrine trophic support, neural stem/progenitor cells (NSC) or mature neurons can act as more direct neural replacements. With a wide therapeutic time window after stroke, biotherapies could be used to treat many patients. However, guidelines for selecting the optimal time window, and the best delivery routes and doses are still debated and the answers may depend on the chosen product and its expected mechanism including early neuroprotection, delayed neural repair, trophic systemic transient effects or graft survival and integration. Currently, the great variety of growth factors, cell sources and cell therapy products form a therapeutic arsenal that is available for stroke treatment. Their effective clinical use will require prior careful considerations regarding safety (e.g. tumorigenicity, immunogenicity),...
potential efficacy, cell characterization, delivery route and in vivo biodistribution. Bone marrow-derived cell populations such as mesenchymal stromal/stem cells (MSC) or mononuclear cells (MNC), umbilical cord stem cells and NSC are most investigated notably in clinical trials. Finally, we discuss perspectives concerning potential novel biotherapies such as combinatorial approaches (growth factor combined with cell therapy, in vitro optimization of cell products, or co-transplantation) and the development of biomaterials, which could be used as injectable hydrogel scaffold matrices that could protect a cell graft or selectively deliver drugs and growth factors into the post-stroke cavity at chronic stages. Considering the remaining questions about the best procedure and the safety cautions, we can hope that future translational research about biotherapies will bring more efficient treatments that will decrease post-stroke disability for many patients.

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RÉSUMÉ

Les accidents vasculaires cérébraux (AVC) représentent la seconde cause de mortalité et la première cause de handicap. En améliorant la neuroprotection et la plasticité endogènes, fréquemment insuffisantes pour assurer une récupération complète, les biothérapies semblent très prometteuses. Leurs mécanismes d’action dépendent du facteur de croissance ou des cellules choisis. On peut distinguer un effet neurotrophique paracrine pour les cellules souches « périphériques » et un effet de remplacement cellulaire plus direct pour les cellules souches neuronales (NSC). Cependant, les délais, les doses et les voies d’administration restent débattus. Parmi les nombreuses cellules disponibles, les cellules de la moelle osseuse ou du cordon (par ex : cellules souches/stromales mésoenchymateuses, ou cellules mononucléées) et les NSC sont les plus étudiées. En perspective, nous discutons ici les thérapies combinées (facteur de croissance et cellules, co-transplantations…) et l’utilisation de biomatériaux (hydrogels injectables) libérant l’agent thérapeutique ou protégeant le greffon. En tenant compte des questions en suspens sur la meilleure procédure et la sécurité (par ex : tumorigénicité, immunogénicité), nous pouvons espérer que les biothérapies deviennent une stratégie thérapeutique efficace, avec une large fenêtre thérapeutique, pour réduire le handicap post-AVC de nombreux patients.

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1. Introduction: pathophysiology of stroke recovery

Stroke is the second leading cause of death worldwide with more than six million deaths per 17 million strokes each year. Stroke has an incidence from 1 to 4/1000/year with 31% occurring before the age of 64 [1]. Moreover, it is the most common source of severe disability in adults affecting 60–70% of stroke survivors. Ischemic strokes represent around 80% of all cases. Excepting early thrombolysis by alteplase (tPA), craniectomy for large strokes or admission to a stroke unit for intensive care, no treatment currently exists to efficiently enhance recovery after stroke [2]. Therefore, the development of new therapies that can enhance brain remodeling processes is crucial and success in this endeavor requires a pathophysiological viewpoint [3–6]. It is well-known that stroke effects are not limited to neurons but involve both brain cells and the surrounding extracellular matrix in a “glio-neurovascular niche” that interacts with the peripheral immune system [7–11]. For these reasons, new therapies should target all these systems rather than narrowly targeting an individual damage process, perhaps then avoiding the failures of past clinical translational attempts to develop specific neuroprotective drugs.

Biotherapies for stroke, including growth factors and cell-based therapies, could achieve the goal of broad physiological action by simultaneously promoting both endogenous neuroprotection and neural repair, including neurogenesis, angiogenesis, oligodendro-gliogenesis, axonal sprouting, and synaptogenesis [4,12–17]. These approaches also have the advantage of action over an extended therapeutic time-window after stroke and thereby might be effective in more patients than those helped by current acute strategies such as thrombolysis or neuroprotectant treatments.

1.1. Endogenous neural protection after stroke

Early after stroke, in addition to possible reperfusion via collateral arteries or fibrinolysis, several endogenous protective mechanisms are spontaneously engaged [18] (Fig. 1). During the acute phase, there is activation of “anti-excitotoxic” channels, receptors or regulators such as γ-aminobutyric acid (GABA) or K⁺ receptors. These phenomena are followed by a transduction phase with amplification of kinases, transcription factors and growth factors (neurotrophin 3, granulocyte colony-stimulating factor [GCSF], vascular endothelial growth factor [VEGF], brain derived neurotrophic factor [BDNF]…). Finally, anti-apoptotic (heat shock protein [HSP 70], BCL2, inhibitors of apoptosis proteins [IAP],
hypoxia inducible factor (HIF1)…, anti-inflammatory (IL10, transforming growth factor β1 [TGFβ1]…) and anti-oxidative (α-tocopherol, vitamin C) molecules stimulate neural repair processes [19]. For example, in astrocytes, HIF1 notably induces erythropoietin production and then activates neuroprotective and neuro-angiogenic phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway [20].

1.2. Post-stroke plasticity and neural repair

The adult brain has a striking capacity for self-repair (Fig. 1) often resulting in re-emergence of childhood organizational patterns [21]. During stroke recovery, beyond phenomena related to adaptive functional compensation, there is “structural” brain plasticity [22,23] based on the surviving tissue participating in reorganization of damaged networks [24,25], and exhibiting neuro-synaptogenesis with axonal sprouting that can persist for several months [26–29]. This form of post-stroke plasticity notably occurs in elderly human brains [30–32]. Stroke also increases neurogenesis from neural stem cells (NSC) of the subventricular zone (SVZ) and hippocampal dentate gyrus, generating neuroblasts that migrate to the lesion and differentiate into mature neurons [29,33]. This post-stroke neurogenesis is closely linked to angio-vasculogenesis and glial function leading to the concept of a “glio-neurovascular niche” [11,34–36] as a favorable “stem cell niche” [37]. Endothelial cells, but not vascular smooth muscle cells, release factors that both stimulate the self-renewal of NSC and also enhance neuron production [38]. After stroke, microvessels size and density changes [39,40] stimulate the neuronal plasticity [41]. For several months following stroke, neuroblasts from the SVZ migrate close to vessels through an area exhibiting early vascular remodeling [42] stimulated by the release of neurotrophic factors such as angiopoietin 1 (Ang1) [43], stromal-derived factor (SDF1) [34], BDNF [44], VEGF [45,46] or metalloproteases [47]. Neuroblasts also enhance angiogenesis with the release of VEGF [48], a relationship underlining the bidirectional link between microvascular and neuronal remodeling.

Additionally, glial cells play a key role during post-stroke recovery, with astrocytes removing excitatory neurotransmitters (glutamate) and K⁺ thereby limiting excitotoxic damage. These cells also modulate synaptogenesis by enhancing the formation of functional synapses [49]. After injury, astrocytosis is linked to microvessels [50] and has a beneficial effect on NSCs via the sonic hedgehog pathway. Otherwise, oligodendrocyte progenitors that are resident in white matter or derived from SVZ NSCs differentiate after stroke into mature oligodendrocytes, thereby enabling axonal remyelination and strongly contributing to brain repair processes [36,51,52]. Microglia also contribute to this post-stroke remodeling
2. Neuroprotective drugs for stroke: lessons for translational research

Most of the known acute damage processes have been targeted by one or more studies utilizing neuroprotectants [5, 59] (Fig. 1). Despite thousands of positive results from animal experiments, it has been particularly disappointing that no clinical trial has demonstrated a clear benefit for any neuroprotective drug after stroke [60, 61]. The drugs that have been investigated can be sorted in several types: anti-excitotoxics targeting glutamate toxicity [62], calcium-blockers [63], antioxidants [64, 65], anti-apoptotics [66], angiotensin receptor blockers (sartans), prostaglandin receptor antagonists [67, 68], anti-inflammatory or immunomodulators [69] such as cyclosporine A [70], immunotherapy to limit the TPA neurotoxicity [71, 72], stimulants (amphetamine), or anti-edema agents (mannitol). Finally, some neuroprotective strategies have exhibited several or unclear effects. These include: induced hypothermia [73–75], hyperbaric or normobaric oxygen therapy [76], albumin [77], magnesium [78], the anti-aggregant cilostazol [79], statins [80], citicoline [81], sildenafil [82], or a treatment based on traditional Chinese medicine (MILC01, Neuroaid) [83].

Despite the past failure of their transfer to clinical environments, neuroprotective therapies are still actively investigated. In this context, some recent findings should be noted, such as the study utilizing the post-synaptic protein PSD95 inhibitor in monkeys [84]. Moreover, encouraging data about stroke recovery were obtained in clinical trials assessing fluoxetine, a serotonin reuptake inhibitor [85], edaravone, a free radical scavenger [65, 86], or minocycline [87].

Lessons from past clinical trial failures should be carefully considered in designing future translational research stroke recovery studies, as the experience gained in these previous trials may be profitably inform the new trials and increase the chance of successful clinical biotherapy transfers [88]. Although rodent models allow convenient exploration of a range of putative pathophysiological mechanisms and potential therapies, the anatomic and pathophysiologic differences between rodent and human stroke arising from differences in cortical architecture, regional neuronal density, arterial network distribution, immunological mechanisms, pre-existing vascular risk factors, concomitant medical treatments, and recovery duration intervals could easily impede the effective translation of animal study results to the clinic.

The effect of these inter-species’ differences on clinical transfer potential might be anticipated by using computational modeling techniques. “In silico” mathematical stroke models can simulate neuroprotective drug [89] or cell therapy effects by implementing explicit models of neurogenesis [90]. The development of these models could facilitate the generation of mechanistic hypotheses that could enhance subsequent clinical transfer of promising treatments. In addition, effort could profitably be exerted to improve experimental methods and statistical data analysis of neuroscience preclinical experiments [91]. For this purpose, preclinical studies may be conducted following clinical trial guidelines, incorporating randomized treatment assignment, blinded assessments, multicenter design, and carefully defined inclusion and non-inclusion criteria [92, 93]. Modeled on the format of the Stroke Therapy Academic Industry Roundtable (STAIR) [94], the stem cell therapeutics as an emerging paradigm in stroke (STEPS) consortium guidelines created in 2007 [95–98] summarize useful recommendations to enhance the translational development of biotherapies.

3. Growth factor treatment in stroke

Numerous growth factors have been investigated as potential regenerative biotherapies for stroke, particularly during the post-acute phase [15–17] (Fig. 1). Unfortunately, as neuroprotectants, none of them showed clear benefits in patients. Growth factors target enhancement of brain remodeling by either mobilizing endogenous stem/progenitor cells, such as cytokines like GCSF or chemokines like SDF1, or by direct trophic effects acting to support damaged neurons, as in the effects of BDNF or glial-derived neurotrophic factor (GDNF).

3.1. Colony stimulating factors

Colony stimulating factors, such as hematopoietic growth factors, can mobilize the release of bone marrow stem cells into the circulation and result in neuroprotective effects in experimental stroke [99]. Although erythropoietin [100] improves neuronal survival, angiogenesis, axonal sprouting, inflammation [101] and oligodendrogliogenesis [102], it appears to increase mortality in clinical trials [103]. Moreover, sequential administration of erythropoietin and β-human chorionic gonadotropin failed to show a benefit despite good safety results during their pilot clinical trial [104]. Concerning GCSF, its experimental effects on neural repair remains controversial [105, 106] as it could increase macrophage infiltration into ischemic brain regions [107]. Combining GCSF and stem cell factor (SCF) treatment could enhance post-stroke angiogenesis, neuronal survival and functional outcome [108]. However, GCSF injection alone did not provide any significant clinical benefit during the acute (<9 h) [109] or subacute (<48 h) phase after stroke [110].

3.2. “Neurotrophic” growth factors

In the same manner, administration of neural angio-trophic supports such as SDF1 [111, 112], BDNF [44, 113, 114], GDNF [115], angiotropin [116–118], VEGF [119–121], fibroblast growth
factor (FGF) [122,123], or hepatocyte growth factor [124] have not yet been evaluated in clinical trials despite encouraging results from preclinical studies. Thus, additional studies are warranted to further evaluate the use of growth factors as useful stroke treatments. In the future, an interesting strategy would be the combination of growth factors with cell therapy or biomaterials, as discussed below in “Perspectives concerning the use of biotherapies for stroke”.

4. Cell therapy after stroke

Cell-based therapies are particularly relevant as neurorestorative treatment for stroke [4,12–17,125–130]. With a wide therapeutic time-window, they could be used to treat many stroke patients, potentially generating significant increments in societal value [131]. Transplanted cells, an example of “plastic” biological products, can adapt to different local conditions in damaged brain tissue while not being limited to a unique target. They can act on a wide range of endogenous neuroprotective and neural repair processes including immunomodulation, neuronal, vascular and glial remodeling (Fig. 1). Traditionally, we distinguish two main treatment strategies, either paracrine trophic support using “peripheral” stem or stromal cells, or direct neural replacement using neural stem/progenitor cells or mature cells such as neurons. The route, dose, and timing for cell transplantation after stroke are still debated, depending on the chosen cell product and the expected therapeutic effect.

4.1. Cell sources and therapeutic cells

Today, the great variety of available cell types and sources form a rich therapeutic arsenal for stroke which requires, prior to clinical use, careful consideration regarding their respective preclinical safety and efficacy profiles, cell characterization, mechanisms of action, delivery routes and in vivo biodistribution properties [125,132]. Currently, among the many available cell types, bone marrow-derived cell populations such as mesenchymal stromal/stem cells (MSC), umbilical cord stem cells and neural stem cells (NSC) are the most commonly investigated in clinical trials.

4.1.1. Cell sources

Cell sources can be sorted by their adult, fetal (extra-embryonic), or in vitro cell-culture origin [133]. While adult sources, such as bone marrow [134–136], are widely used in clinical trials (Table 1), adipose tissue containing MSC [137–140], peripheral blood [141–144], olfactory mucosa [145], menstrual blood [146], brain tissue [147], breast milk [148] or dental tissue [149] are interesting alternatives for neurorestorative therapy. “Fetal” sources, such as umbilical cord, are relatively easy to collect for banking and can provide cell products for stroke therapy from either the cord itself (Wharton’s jelly) [150] or from cord blood samples [151]. Placenta [152,153], amniotic fluid [154], or fetal brain samples, including striatum or 1st trimester cerebral cortex [155,156] are already used as cell sources in current clinical trials. Finally, in vitro sources such as NSCs [157] or neural cell cultures [155,158] have already been used in clinics. From in vitro cultures, pluripotent cells such as embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) from adult tissue cannot be directly used due to their associated high tumorigenicity risk [159]. However, as sources, ESC [160–162] and iPSC [163,164] can be expanded over many passages, thereby providing a virtually unlimited supply of multipotent or mature derived cells usable for cell therapy.

4.1.2. Cell therapy products

Cell therapy products harvested from these different sources can be used for xenogenic, allogenic or autologous treatments. Three main therapeutic cell categories can be distinguished:

- mesoderm-derived stromal or stem cells;
- ectoderm-derived neural stem/progenitor cells;
- hematopoietic/endothelial stem cells.

Stromal/stem cells isolated from bone marrow, umbilical cord, blood or adipose tissue are widely used in cell therapy trials for stroke. MSC and multipotent adult progenitor cells (MAPC) can be used under autologous or allogenic conditions without concomitant immunosuppressive drugs owing to their immunomodulatory properties [135,136,165,166]. For MSC, precautions must be taken when using ex vivo cultures. Indeed, excessive MSC expansion with several passages could affect their therapeutic features [167] and one study suggest that cell culture media, either fetal bovine serum or autoserum, would modify the final MSC phenotype [168]. Mobilized into blood from bone marrow after stroke, very small embryonic-like stem cells require further study to assess their therapeutic potential for stroke [142].

The second category includes neural stem/progenitor cells (NSC) harvested from brain tissue, immortalized neural cell lines or cells derived from ESC or iPSC cultures [155,169,170]. NSC grafts provide functional benefit but usually require immunosuppressant treatments. An immortalized neural cell line (ReNeuron) is currently being studied in a clinical trial for stroke in the United Kingdom [171]. We can also include in this heterogeneous “NSC group” the olfactory ensheathing cells [149], oligodendrocyte progenitors [172], and immature neurons [158,173].

The third category of therapeutic cells employed in stroke studies includes the hematopoietic stem cells (HSC CD34+) [174,175] and endothelial progenitors (EP CD34+) [176]. They are easily harvested from cord blood, bone marrow or peripheral blood after mobilization and represent a type of mononuclear cells (MNC) usable, without amplification, to enhance microvascular repair [177,178]. Despite unclear cell characterization, MNC have been used in several clinical trials with encouraging results particularly as a treatment applied a few days after stroke onset (Table 1). Additionally, mature endothelial cells could be an interesting and well-characterized product improving post-stroke vasculogenesis and neurogenesis [147].

4.2. Mechanisms of action

The functional benefits of cell therapy on stroke recovery are well established in animal models using the above-mentioned cell types coupled with different delivery routes [179–181].
<table>
<thead>
<tr>
<th>Reference</th>
<th>Location</th>
<th>Patients (age)</th>
<th>Source</th>
<th>Cell type</th>
<th>Delay stroke to treatment</th>
<th>Dose</th>
<th>Route</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kondziolka et al, 2000 [173]</td>
<td>Pittsburgh USA</td>
<td>12 IS (61 years, 44–74 years)</td>
<td>Allo/Human embryonic teratocarcinoma cell line (NT2/D1)</td>
<td>LBS-neurons (Layton BioScience)</td>
<td>2.5 years (7 months–4.5 years)</td>
<td>2 (60 µL; n = 8) or 6 million (180 µL; n = 4) + Cyclo. A (9 weeks)</td>
<td>IC</td>
<td>Feasible Safe (single seizure at 6 months, n = 1; remote stroke at 5 months, n = 1)</td>
</tr>
<tr>
<td>Kondziolka et al., 2005 [158]</td>
<td>Pittsburgh, Stanford USA</td>
<td>6 IS/8 ICH (58 years) 4 controls (3 IS/1 ICH; 46 years)</td>
<td>Allo/NT2/D1</td>
<td>LBS-neurons (Layton BioScience)</td>
<td>3.5 years (1–5 years)</td>
<td>5 (n = 7) or 10 million IC (n = 7) (250 µL) + Cyclo. A (6 months)</td>
<td>IC</td>
<td>Feasible Safe (single post-operative seizure, n = 1; asymptomatic SDH, n = 1)</td>
</tr>
<tr>
<td>Savitz et al., 2005 [256]</td>
<td>Boston USA</td>
<td>5 IS (25–52 years)</td>
<td>Xeno/Primordial porcine striatum + antiMHC1</td>
<td>LGE cells (Genvec)</td>
<td>5 years (1.5–10 years)</td>
<td>1 million/cm^3 of infarct 50 (n = 4) or 80 million (n = 1)</td>
<td>IC</td>
<td>Feasible Adverse events (cortical vein occlusion, n = 1; hyperglycemic seizures, n = 1; both with MRI transient abnormalities) FDA termination</td>
</tr>
<tr>
<td>Rabinovitch et al., 2005 [257]</td>
<td>Novosibirsk Russia</td>
<td>7 IS/3 ICH (46 years; 35–56 years) 11 controls (6 IS/5 ICH; 55 years)</td>
<td>Allo/&quot;immature&quot; nervous and hemopoietic tissues</td>
<td>Immature cells (NSC?)</td>
<td>4–24 months</td>
<td>1 injection (n = 5) or 2 injections (n = 5)</td>
<td>IThec (LP)</td>
<td>Feasible Safe (transient meningeal syndrome)</td>
</tr>
<tr>
<td>Bang et al., 2005 [258]</td>
<td>Suwon South Korea</td>
<td>5 IS (63 years; 54–72 years) 25 controls (59 years)</td>
<td>Auto BM</td>
<td>MSC Expansion in FCS</td>
<td>4-5 and 7-9 weeks</td>
<td>50 million × 2</td>
<td>IV</td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>Lee et al., 2010 [260]</td>
<td>Suwon South Korea</td>
<td>16 IS (65 ± 14 years) 36 controls (64 ± 12 years) including the previous trial</td>
<td>Auto BM</td>
<td>MSC Expansion in FCS</td>
<td>2.5–5 and 5–9 weeks</td>
<td>50 million × 2</td>
<td>IV</td>
<td>Feasible (fever after first injection, n = 1: no second injection) Safe (3–5 year follow-up) Beneficial: recovery, increase of serum SDF1α</td>
</tr>
<tr>
<td>Mendonça et al., 2006 [264]</td>
<td>Rio de Janeiro Brazil</td>
<td>1 IS (54 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>4 days</td>
<td>300 million (3 mL/10 min)</td>
<td>IA</td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>Suarez-Monteagudo et al., 2009 [263]</td>
<td>Habana Cuba</td>
<td>5 IS (?)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>1–10 days</td>
<td>?</td>
<td>IC</td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>Battistella et al., 2011 [265]</td>
<td>Rio de Janeiro Brazil</td>
<td>6 IS (24–65 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>2–3 months</td>
<td>125–500 million including 20 million 99mTc-labeled</td>
<td>IA</td>
<td>Feasible Safe Biodistribution: brain, liver, lungs, spleen, kidneys</td>
</tr>
<tr>
<td>Barbosa et al., 2010 [272]</td>
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<td></td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>Savitz et al., 2011 [268]</td>
<td>Houston USA</td>
<td>10 IS (55 ± 15 years) 79 historical controls (63 ± 12 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>1–3 days</td>
<td>7 (n = 1) or 8.5 (n = 1) IV or 10 million/kg</td>
<td>IV</td>
<td>Feasible Safe (death/pulmonary embolism at 40 days, n = 1)</td>
</tr>
<tr>
<td>Honmou et al., 2011 [259]</td>
<td>Sapporo Japan</td>
<td>12 IS (59 ± 8 years, 41–73 years)</td>
<td>Auto BM</td>
<td>MSC Expansion in auto serum</td>
<td>10 weeks (5–19 weeks)</td>
<td>110 million (60–160 million)</td>
<td>IV</td>
<td>Feasible Safe (transient fever, n = 1)</td>
</tr>
<tr>
<td>Reference</td>
<td>Location</td>
<td>Patients (age)</td>
<td>Source</td>
<td>Cell type</td>
<td>Delay stroke to treatment</td>
<td>Dose</td>
<td>Route</td>
<td>Results</td>
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<tr>
<td>Bhasin et al., 2011 [261]</td>
<td>New Delhi India</td>
<td>4 IS/2 ICH (42 years, 20–59 years) 6 controls (5 IS/1 ICH; 46 years)</td>
<td>Auto BM</td>
<td>MSC</td>
<td>Expansion in animal serum-free media</td>
<td>9 months (7–12 months)</td>
<td>50–60 million</td>
<td>IV</td>
</tr>
<tr>
<td>Bhasin et al., 2013 [262]</td>
<td>New Delhi India</td>
<td>18 IS/2 ICH (45 ± 12 years) 20 controls (19 IS/1 ICH; 45 ± 10 years) including the previous trial</td>
<td>Auto BM</td>
<td>MSC: expansion in animal serum-free media MNC</td>
<td>10 months (3 months–2 years)</td>
<td>MSC: 50–60 million (n = 6) MNC: 50–60 million (n = 14)</td>
<td>IV</td>
<td>Feasible Safe Beneficial: recovery (for MNC), activation fMRI</td>
</tr>
<tr>
<td>Prasad et al., 2012 [269]</td>
<td>New Delhi India</td>
<td>11 IS (30–70 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>7–30 days</td>
<td>80 million</td>
<td>IV</td>
<td>Feasibility = 11/11 (target-dose = 9/11) Safe</td>
</tr>
<tr>
<td>Moniche et al., 2012 [266]</td>
<td>Seville Spain</td>
<td>10 IS (67 ± 14 years) 10 controls (67 ± 13 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>6 days (5–9 days)</td>
<td>159 million</td>
<td>IA</td>
<td>Feasible Safe (seizure at 3 months, n = 2) Increase of serum bNGF</td>
</tr>
<tr>
<td>Friedrich et al., 2012 [267]</td>
<td>Porto Alegre Brazil</td>
<td>20 IS (?)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>3–7 days</td>
<td>220 million</td>
<td>IA</td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>Li et al., 2013 [270]</td>
<td>Shandong China</td>
<td>60 ICH (56 years, 39–74 years) 40 controls (56 years, 35–72 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>6 days (5–7 days) (3.5 mL)</td>
<td>2.4–23 million</td>
<td>IC</td>
<td>Safe (transient fever, n = 5; lung cancer, n = 1) Beneficial</td>
</tr>
<tr>
<td>NCT01028794 (Taguchi 2008–13)</td>
<td>Osaka, Kobe Japan</td>
<td>12 IS (20–75 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>7–10 days</td>
<td>Full from 25 mL of BM Full from 50 mL of BM</td>
<td>IV</td>
<td>Feasible Safe</td>
</tr>
<tr>
<td>NCT00473057 (Andre et al., 2005–11)</td>
<td>Rio de Janeiro Brazil</td>
<td>12 IS (18–75 years)</td>
<td>Auto BM</td>
<td>MNC</td>
<td>&lt; 90 days</td>
<td>500 million 500 million</td>
<td>IA IV</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT00950521 (Lin 2009–10)</td>
<td>Taichung Taiwan India*</td>
<td>30 IS (35–70 years) including controls 120 IS (18–70 years) including controls</td>
<td>Auto blood</td>
<td>CD34+ SC</td>
<td>6–60 months</td>
<td>2–8 million</td>
<td>IC</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT01501773 (INVEST, Prasad 2008–11)</td>
<td>Chattanooga USA</td>
<td>44 IS (18–80 years) including controls</td>
<td>Allo placenta</td>
<td>Placenta derived SC (Celgene PDA001)</td>
<td>Subacute</td>
<td>200 million 200 million × 2 800 million × 2 (interval = 7 days)</td>
<td>IV</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Sorted by date (black: intracerebral [IC], red: intra-arterial [IA], or blue: intravenous [IV] cell-delivery route studies). Keywords on PubMed-NCBI and clinicaltrials.gov: “cell therapy” or “stem cell” or “transplantation” or “bone marrow” AND “stroke” or “cerebral ischemia”.

Allo: allogeneic; Auto: autologous; BM: bone marrow; Cyclo: cyclosporine; FCS: fetal calf serum; ICH: intracerebral hemorrhage; IThec (LP): intrathecal (lumbar puncture); IS: ischemic stroke; LGE: lateral ganglionic eminence; MHC: major histocompatibility complex; MNC: mononuclear cells; MSC: mesenchymal stromal/stem cells; βNGF: nerve growth factor; NSC: neural stem cells; SC: stem cells; SDF1α: stromal cell derived factor 1α; SDH: subdural hematoma; Xeno: xenogeneic

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However, their respective mechanisms of action are complex and vary according to the transplanted cell type [13,14,132]. A common characteristic of these cell products is that they simultaneously target many different host brain cell types, including NSCs, neurons, endothelial and glial cells, leading to improvements via several endogenous repair processes, such as neuro-glio-angiogenesis, axonal sprouting, synaptogenesis. Direct replacement of injured neurons ("homotopic" repair) has been suggested after NSC IC [182,183] or IA injection [184] encouraging possible long-term survival of implanted neurons in human [185], after iPSC derived neuron [186], bone marrow cell [187] or ESC-derived MSC injection [160]. However, only a few grafted cells can be expected to express neuronal markers, so long-term graft survival is relatively poor [188–192]. Moreover, despite possible integration of grafted NSCs [193–195] into the host circuitry, functional recovery occurs too early to be caused by newly formed neurons and synapses. Additionally, as emphasized for IV-injected cord blood cells, cell entry or integration into the host brain would not be required to obtain neural repair enhancement [196,197]. Concerning HSC, the possibility of their efficient differentiation into neurons is still debated [198–200]. Direct replacement of all damaged brain cells, including cells from ectoderm such as neurons or glia, and mesoderm such as microglia or endothelium, would require transplantation of "native" pluripotent cells (ESC or iPSC) which might result in tumor formation [161].

Thus, it seems more realistic to expect that cell therapy, notably employing "non-neural" cells, such as MSC or MNC, works through paracrine trophic support ("by stander") effects on the injured brain by secreting various growth factors [201,202]. The improvement in host brain plasticity and associated recruitment of endogenous progenitors has been identified after injection of MSC [203] via the sonic hedgehog pathway [204], NSC [157] notably by enhancing dendritic plasticity [205], or olfactory ensheathing cells [145]. Moreover, the effects of cell therapies on post-stroke vasculogenesis and angiogenesis seem to be crucial in explaining early post-graft benefits. IC injection of endothelial cells can improve vasculogenesis linked to neurogenesis via VEGF release mechanisms [147]. Proangiogenic effects were also observed early after injection of MSC [206–209] that can contribute to VEGF-induced angiogenesis by supplying metalloprotease MMP-9 [210], after injection of NSC [211,212], EP [213], or cord-blood MNC CD34+ [175]. These MNCs contain EP and smooth muscle progenitors which may collaborate to form a mature vascular network supporting and enhancing neuroblast survival and migration after stroke [214]. Moreover, EP, MSC or NSC could also facilitate protection or restoration of the blood-brain barrier after stroke [126,211,215].

Another important effect of cell therapy is enhanced glial remodeling and limitations in anterograde degeneration [216–218]. For example, IV injection of MSC has beneficial effects on both post-stroke glial remodeling and axonal remyelination [219]. It also increases GDNF levels, creating a hospitable environment for neural repair and neuroblast migration from the SVZ [220].

Finally, cell therapies can also limit host cell death through anti-apoptotic [153,221,222] and immunomodulatory mechanisms. Although MSCs are known to attenuate microglia and leukocyte inflammatory responses after stroke [223–225], some immunomodulation properties were also observed for cord blood cells [226] or NSC [227,228], which can both influence splenic inflammatory responses after stroke [229].

### 4.3. What is the optimal route for cell delivery?

The best combination of delivery route and dose for cell therapy after stroke still remains debated, depending strongly on the chosen cell products and expected therapeutic effects. Theoretically, we can expect that intracerebral (IC) delivery would be preferred for cell replacement while intravenous (IV) or intra-arterial (IA) injection would be better for systemic and trophic support. Systemic intravascular injections are less invasive and easier to implement than surgical implantation, properties that are relevant in stroke therapy [230]. This administration route allows cell distribution into vascularized and viable areas of the lesion, not only into localized graft sites. Because cytokine, chemokine and inflammatory signals are released by compromised brain tissue [231,232] such as SDF1/CXCR4 [233], IV-injected MSCs can preferentially migrate to the damaged brain regions despite initial and transient lung trapping [234]. In a model of hemorrhagic stroke, compared to IC injection, only IV-injected NSCs were found to have an efficient anti-inflammatory effect, measured by the degree of attenuation of splenic activation of tumor necrosis factor-alpha (TNF-alpha), interleukin 6 (IL6), and nuclear factor-kappa B (NF-kB) [229]. In the same manner, cord blood cells were more effective after IV compared to IC administration [235], a result contrary to other studies showing poor efficacy associated with use of the IV route [236,237]. As only 1 to 4/10,000 IV-injected cells reach the arterial system and the brain tissue targets (1 to 4/10,000 IV-injected cells) [234], the acute pulmonary passage after IV injection is a major obstacle for IV delivery of NSCs, MAPCs and particularly for larger cells such as MSCs [238]. This pulmonary passage can be increased by inhibition of MSC CD49d, infusion via two boluses [238], or sodium nitroprusside preadministration [239].

Other graft routes, such as IA [190,240,241] or IC [236,237,242,243], could avoid the lung entrapment problem and thereby increase the number of grafted cells in the target tissue. 111In-labeled NSCs can be detected in the ischemic hemisphere after IA but not IV injection [244]. This is consistent with an in vivo MRI study showing brain penetration of MSCs only after IA injection [245]. However, this study reported a risk of vascular occlusion after IA cell delivery, which could be linked to the large size of MSCs [246]. For NSC delivery, although the IA route seems to be efficient [247], it can increase mortality [248]. To continue the debate, it was recently reported that IV and IA injection of MNCs lead to the same benefit [249]. Thus, whereas IA or IC grafts are feasible administration routes for stroke therapy, IV cell injection has been selected for the majority of clinical trials (Table 1). Further discussion can be seen in the “Clinical trials of cell therapy for stroke”.

### 4.4. What is the optimal therapeutic time-window?

As in route and dose, the optimal timing for cell transplantation after stroke remains debated, depending on the cell type,
allowing a potential delay for in vitro amplification, and their specific mechanism of action, involving acute neuroprotection vs. delayed neural repair, or trophic systemic transient effects vs. graft survival or integration \[230\] (Fig. 1). For example, early (6–24 h post-stroke) IA injection of NSCs leads to greater differentiation into astrocytes, whereas injection at later time points (7–14 days post-stroke) leads to greater differentiation into neurons \[184\]. MNCs, obtained from bone marrow or cord blood without amplification delay, could be good candidates for acute delivery in the first 72 hours after stroke \[177,197,250\], possibly serving as an adjunct to thrombolysis or mechanical thrombectomy \[251\].

A great clinical advantage of cell therapies would be to delay stroke treatment until the post-acute and rehabilitation phases in a majority of patients. Whereas some studies suggested that only early (3–24 h) IV injection of MSCs could be effective \[252\], others have shown that treatment delays up to one month after stroke can also result in improvements in recovery \[192,253\]. Give up to one month after experimental stroke, IA transplantation of immortalized neurons \[254\] or IV injection of umbilical cord derived cells \[222\] both enhance recovery. While a 6-week-transplantation delay in NSC administration does not influence cell migration, proliferation or neuronal differentiation, it could result in poorer graft survival compared to early transplantation (48 h) \[183\].

Thus, the issue of optimal route, dose, and time-window for cell therapy persists. Different cell products can be used at different time points. Further investigations that take into account the difference between animal models and human stroke in terms of recovery timing and duration are clearly warranted.

5. **Clinical trials of cell therapy for stroke**

Seventeen clinical trials have been published and 5 others are complete but not yet published (Table 1). The cell source was most commonly autologous bone marrow (n = 16 trials), followed by NSC/neuron cultures (n = 4), and autologous peripheral blood (n = 1) or placenta (n = 1). Several cell products and routes were investigated: IC or intrathecal transplantation of NSC or immortalized neurons \[158,173,255–257\], IV injection of autologous MSC \[258–262\], IC \[263\], IA \[264–267\] or IV \[268,269\] injections of autologous MNC, IC transplantation of HSC/EP CD34+ from autologous blood (Lin, NCT00950521), and IV injection of placenta derived stem cells (Celgene, NCT01310114). Only one unpublished trial directly compared IA vs. IV injection of autologous MNC (Andre et al., NCT00473057). Recently, one trial focused on intracerebral hemorrhage using IC injection of MNC from autologous bone marrow \[270\].

Direct comparisons are difficult due to the important differences in cell products, routes and post-stroke delays among the trials. However, these first studies of cell therapy for stroke have reported encouraging results regarding safety and feasibility \[271,272\]. From the available studies, we can conclude that:

- MSC or MNC (including HSC/EP) injections are easier to employ with possible monitoring of IAInjected MNCs \[273\].

Currently, around 30 on-going trials utilize MNC or MSC as cell products and are exploring the potential utility of allogenic administration and of using umbilical cord blood as the cell source. New biomarkers of stroke recovery, such as multimodal MRI \[274,275\] and biological blood markers, such as SDF1, BNGF or circulating EF, will provide additional important data about cell therapy functional effects and its mechanism of action in human stroke.

6. **Perspectives concerning the use of biotherapies for stroke**

6.1. **Careful translation**

For the development of neurorestorative therapies for stroke, additional translational studies should be conducted regarding the influences related to the stroke type and localization, the usual neurovascular risk factors, such as hypertension, diabetes, and cerebral small artery disease, and comitant treatments, such as tPA thrombolysis or statins. Lesion location and size will be important factors to determine which patients are suitable for cell therapy. For example, IV MSC could be less efficient in treating stroke patients with a lesion including the SVZ \[260\]. Concerning stroke type, little data are available concerning hemorrhagic stroke. Despite benefits seen in experimental studies of MSC IA or IV injections \[139,241\], it remains unclear if neurorestorative therapy clinical trials should include both hemorrhagic and ischemic strokes regarding differences in pathophysiology and recovery. Concerning risk factors, recent studies have suggested that IV injection of MSC given 24 h post-stroke induces adverse effects in diabetic rats increasing blood-brain barrier leakage and vascular damage via increased expression of angiogenin \[276\]. Negative results were also reported in hypertensive rats after acute IV injection of bone marrow MNCs \[277\] or cord blood cells \[278,279\]. Otherwise, the combination of simvastatin and cord blood cell IV injection 24 h post-stroke increased BDNF/TrkB expression, enhanced cell migration towards the ischemic brain tissue, and amplified neuro-synaptogenesis, improving recovery \[280\].

6.2. **Safety issues related to tumorigenicity and immunogenicity**

In considering translation study characteristics, beyond determining the presence of good cell characterization, safety concerns must be emphasized. While tumorigenicity was clearly identified following injection of “native” ESCs or iPSCs, it could also be a safety issue for MSCs according to their specific source, whether adult bone marrow or umbilical cord \[281\]. Even so, across numerous studies, there has never been a report of tumor emergence, even after long-term follow-up \[190,260\]. However, the first case of a human brain tumor occurred four years after a NSC therapy in a child \[282,283\] indicates that tumorigenicity must be carefully taken into
consideration for all cell therapy products using long-term surveys.

Concerning immunogenicity, we previously discussed the immunomodulation properties of MSCs from autologous or allogenic sources [219]. MSCs or MNCs harvested from human sources are widely used in animal models without immunosuppression. In contrast, for allogeneic NSCs, the use of concomitant immunosuppression is needed [158,171] to avoid severe inflammatory reaction notably after local delivery [284]. Even so, some experimental studies have reported potential “anti-inflammatory” effects associated with NSCs [227–229]. A small trial has indicated that anti-MHC1 pretreatment of porcine NSC cultures is insufficient to fully avoid human host immunoreactivity [256]. Moreover, autologous transplantation of iPSC-derived neurons elicited only a minimal immune brain response, in contrast to allografts, which caused an acquired immune response with a microglia activation and leukocyte infiltration [285]. Thus, the development of iPSC-derived cell therapy will probably require an autologous source, such as skin, or HLA-haplotyped iPSC banking.

In the future, the development of neurorestorative therapies for stroke should carefully take into consideration all these translational and safety concerns to allow an efficient transfer from bench to bedside, particularly for the emerging cell products such as genetically-modified cells.

6.3. Combinatorial approaches: co-treatment, cell modification, co-transplantation

Enhancing the graft survival and cell therapy benefits is the common aim of several on-going experimental strategies, including combinations of neuroprotectants or growth factors with cell therapy, modification of therapeutic cells to increase a specific feature, or co-transplantations of “synergic” cells.

Concerning combinations, we have already noted that statins can increase the benefits seen with IV injection of cord blood cells [280]. Erythropoietin [286] or NO donor [287] could also act in synergistic ways with MSC IV injection, as seen when VEGF is combined with IV injection of NSC [288] resulting in a pro-angiogenic effect, or when GCSF is combined with MNC [289]. Currently, biotechnology allows genetic cell modifications or improvements in cell culture conditions [134] allowing delivery of cell products overexpressing interesting factors. Thus, genetically modified-MSC overexpressing Ang1 [208], BDNF [290,291], GDNF [292,293], VEGF or VEGF-Ang1 [294] can all increase the effects of MSC IV injection on post-stroke neural repair and recovery. Moreover, without gene modification, MSC cultures exposed to hypoxia [295] or co-cultured with depolarized astrocytes [296] could be interesting to respectively enhance their HIF1 and growth factor expression or their neuronal differentiation, thus improving their therapeutic potential after stroke.

Co-transplantation of synergic cells would be another strategy to increase the benefit of cell therapy. For example, IV co-injection of MSC and EP 24 h post-stroke enhance expressions of bFGF, VEGF and BNDF [297]. IV injection of cord blood EP and smooth muscle progenitors 24 h post-stroke, which collaborate to enhance angiogenesis, results in the maintenance of neurogenesis and neuroblast migration to the peri-ischemic cortex [214].

6.4. Biomaterials enhancing neurorestorative therapy in stroke

The combination of drugs, growth factors and cells with biopolymer scaffolds (i.e. matrix) of extracellular matrix molecules, including collagen and hyaluronic acid, may protect the graft and provide localized and controlled delivery of the therapeutic agent [298,299]. The use of such “carrier” scaffolds are particularly relevant for injections into the stroke cavity at a chronic stage, as they might help to avoid a deleterious injection into the adjacent brain tissue where important recovery processes may be underway. Thus, close to the plastic brain regions, grafted scaffolds could provide a favorable environment for drug delivery and graft cell survival.

After stroke, different hydrogel scaffolds have already been used for IC delivery of cyclosporine [300], VEGF [301,302], erythropoietin [303], epidermal growth factor [304], BDNF for enhancing endogenous NSC survival [305], or anti-receptor Nogo to improve neuro-synaptogenesis [306].

For cell therapy, we noted that most grafted cells die after IC graft into the damaged area. To improve graft cell survival, proliferation, migration and differentiation, different hydrogels such as hyaluronic acid or collagen gels, or matrigel have been assessed as cell-seeded scaffolds injected at chronic stages (1–3 weeks post-stroke). The approaches include ESC-derived NSC incorporated into Matrigel [307], embryonic cortex NSC into hyaluronan-heparin-collagen hydrogel [308], MSC into thermoreversible hydrogel [309], cord MSC into gelatin-laminin scaffold [310] and MSC-derived endothelial cells into hyaluronic acid hydrogel [311]. The cell scaffolds protect the grafted cells from the host immune system, form a functional vascular network integrated with the host vascular system [311], prevent glial scar formation and reduce neuroinflammation [310]. Interestingly, after the biodegradation of the scaffold, the remaining grafted umbilical cord MSCs could still survive [310]. However, further optimization of hydrogel compositions is warranted to avoid possible inflammatory responses as observed in immunocompetent mouse brain two weeks after IC injection of a hyaluronic acid hydrogel pre-seeded with human NSCs or glial precursors [312].

Currently, biomaterial researchers are seeking to optimize injectable hydrogels by combining cell seeding with the incorporation of growth factors or tracers. Specific features such as gelation rate, cell adhesion, delivery properties, biocompatibility and degradation must be investigated in experimental studies prior to transferring this promising procedure to clinical trials.

7. Conclusion

Neurorestorative therapies include both growth factors and cell-based techniques that can be combined or used with an injectable biomaterial. They hold great promises for stroke treatment, as they both enhance several processes involved in post-stroke recovery and also can be used across a wide therapeutic time-window. However, preclinical studies must still be conducted in parallel with the clinical trials. Indeed, related to the rapidly expanding therapeutic arsenal of biological treatments, many interdependent
questions remain to be answered about specific mechanisms of action and the details of the procedures themselves. Which growth factor or cell type is most appropriate for patients that may vary in their lesion extent, location, age, or neurovascular risk factors? Which are the optimal dose and delivery routes? What is the therapeutic time-window for which target? Will the expansion of “biological” therapy be associated with increased long-term tumorgenicity and immunogenicity? Assuming that these remaining questions and safety concerns can be adequately addressed, is hoped that the emerging biotherapies will prove to be efficient and effective means to decrease post-stroke disability for many patients.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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