Preconditioning Stem Cells for *In Vivo* Delivery

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Abstract

Stem cells have emerged as promising tools for the treatment of incurable neural and heart diseases and tissue damage. However, the survival of transplanted stem cells is reported to be low, reducing their therapeutic effects. The major causes of poor survival of stem cells *in vivo* are linked to anoikis, potential immune rejection, and oxidative damage mediating apoptosis. This review investigates novel methods and potential molecular mechanisms for stem cell preconditioning *in vitro* to increase their retention after transplantation in damaged tissues. Microenvironmental preconditioning (e.g., hypoxia, heat shock, and exposure to oxidative stress), aggregate formation, and hydrogel encapsulation have been revealed as promising strategies to reduce cell apoptosis *in vivo* while maintaining biological functions of the cells. Moreover, this review seeks to identify methods of optimizing cell dose preparation to enhance stem cell survival and therapeutic function after transplantation.

Key words: aggregate formation; encapsulation; hydrogel; preconditioning; stem cells

Introduction

**Heart and Neural Tissue Damage**, such as myocardial infarction (MI), stroke, or spinal cord injury, are pathological events for which there are no satisfactory treatments to date.1–3 Stem cell therapy has shown promising results for restoration of tissue homeostasis and regeneration of the damaged tissues.4–6 Stem cells or their derivatives have prolonged proliferation ability, multilineage differentiation potential, and trophic functions, which together enhance tissue repair while being integrated in injured tissues.7–9 Therefore, the use of stem cells in cell therapy has been extensively studied recently.

The site of injured tissue is usually associated with ischemia, extracellular matrix (ECM) degradation, oxidative stress, inflammation, and acute immune response.10 As a consequence, stem cells generally display limited survival and low retention rate in injured tissues, reducing the benefit of their therapeutic effects. In addition, stem cell derivatives may tend to be targeted by innate and adaptive immune responses in the host tissue.11 Thus, increasing stem cell retention is required towards long-term cell efficacy *in vivo*. The prolonged survival and integration of stem cell derivatives in damaged tissues rely on the prevention of anoikis, minimization of immune rejection, and increased resistance against oxygen and nutrient deprivation and oxidative stress in the ischemic area.

Recent studies indicate that overexpression of anti-apoptotic and antioxidant proteins provides stem cells the resistance against ischemic stresses, thus increasing their retention *in vivo*.12 However, such methods may have increased risk in long-term integration with the host tissue due to the possibility of tumorigenicity from gene transfection.13 Hence, the development of nongenetic methods is preferred to increase stem cell survival in damaged tissues without further compromising the tissue integrity. It has recently shown that chronic exposure *in vitro* to stresses that cells experience in damaged tissues, such as hypoxia, can enhance stem cell resistance *in vivo*.14 Other approaches are also explored such as heat shock treatment and exposure to hydrogen peroxide to induce heat shock proteins (HSPs) or to increase the resistance against oxidative stress.6,15 In addition to the culture environment preconditioning, growing stem cells in three-dimensional (3D) culture such as formation of 3D aggregates (e.g., microtissues) and the use of hydrogels (e.g., resuspended, embedded, or encapsulated) also provide favorable microenvironments that promote stem cell retention and survival under ischemic conditions due to the intimate cell–cell and cell–matrix interactions and the modulation of trophic functions of stem cells.16,17

Hence, this review analyzes current methods and the possible molecular mechanisms for stem cell preconditioning *in vitro* prior to cell transplantation in injured tissues such as brains and hearts, with the examples of pluripotent stem
cell derivatives, cardiac progenitors, neural progenitors, and mesenchymal stem cells. In particular, this work discusses emerging approaches of preconditioning stem cells through 3D aggregate formation or hydrogel encapsulation to modulate their properties for transplantation study. This survey indicates the feasibility of preconditioning stem cells with enhanced retention and survival, as well as the improved therapeutic functions towards long-term restoration of tissue homoeostasis.

Stem Cells for Therapy

Pluripotent stem cells

Pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) emerge as promising cell sources for tissue engineering and regenerative medicine.18 PSCs have long-term self-renewal ability and a broad potential to differentiate into the cell types of the three germ layers and can in principle provide an unlimited number of cells for transplantation. In particular, iPSCs can be obtained by reprogramming somatic or progenitor cells from the specific patients through the forced expression of pluripotent genes such as KLF-4, c-Myc, Oct-4, and Sox-2,19 enabling possible autologous cell transplantation. While PSCs cannot be directly transplanted in injured tissues due to the risk of teratoma formation,20 tissue-specific progenitor cells or terminally differentiated cells can be derived from PSCs and are more appropriate for cell therapy. To date, oligodendrocyte progenitor cells and retinal pigment epithelium cells are two examples of Phase I clinical trials based on human PSCs for treating patients with spinal cord injury or macular degeneration, respectively.21,22

Mesenchymal stem cells

Many more clinical trials (>300) have been conducted using mesenchymal stem cells (MSCs), which are connective tissue progenitor cells found in perivascular location in vivo (e.g., adventitial reticular cells in bone marrow or satellite cells in muscle).23 MSCs are usually characterized ex vivo by a set of nonspecific markers such as CD73, CD105, and CD90, and the differentiation potential towards osteoblasts, adipocytes, and chondrocytes.24 MSCs can be isolated from various types of tissues including bone marrow, adipose tissue, cartilage, and umbilical cord.25 MSCs have also been derived from PSCs recently through embryoid body (EB) formation and replating in microvascular endothelial cell media.26,27 The derived cells showed the expression of MSC markers and the ability to differentiate into osteocytes, chondrocytes, adipocytes, and myocytes.27 Compared to somatic MSCs, MSCs derived from PSCs have similar biological functions but a reduced telomere shortening process.28 MSCs have been successfully transplanted in vivo, such as in heart tissues in which enhanced cardiac functions were reported.29 The beneficial effects of MSCs mainly rely on the secretion of paracrine factors, such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), or immune-regulatory molecules such as interleukin (IL)-6, IL-10, and indoleamine 2,3-dioxygenase.16,30 However, MSCs generally have limited survival rate and engraftment in vivo due to the cell loss after injection and the hostile environment of injured tissue.31 Thus, increasing MSC retention in vivo should improve and prolong their therapeutic effects.

Neural progenitor or stem cells

Neural progenitor cells (NPCs) exhibit the tri-lineage neural differentiation potential along neurons, astrocytes, and oligodendrocytes, and are usually characterized by the expression of specific markers such as Nestin, SOX-2, and Musashi-1.32 Somatic NPCs can be isolated from adult and fetal tissues (e.g., the subventricular zone and the dentate gyrus of the brain). In addition, NPCs could be derived from PSCs through EB formation or monolayer induction.2 The comparison of somatic and ESC-derived NPCs showed common differentiation potential and secretory profile, but PSC-derived NPCs displayed enhanced proliferation and were less prone to senescence compared to their somatic counterpart.33 Transplantation of PSC-derived NPCs improved the brain or motor functions after stroke, Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and so forth.34,35 The beneficial effects of NPCs in vivo include partial integration with host tissue, the ability to differentiate into neural populations, and the secretion of paracrine factors (such as BDNF) to promote endogenous progenitor differentiation.34 However, the limited engraftment and survival in injured sites are the major hurdles for their therapeutic functions.35

Cardiac progenitor or stem cells

Cardiac progenitor cells (CPCs) can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells, and are usually characterized by the expression of c-Kit, KDR, PDGFR-α, and Nkx2.5.36 CPCs can be isolated from heart tissues or derived from PSCs.36 While somatic CPCs are prone to senescence associated with aging, PSC-derived CPCs can provide an unlimited number of heart cells and be used for constructing cardiac tissues.36,37 CPCs have been successfully infused after myocardial infarction and are able to reduce scar formation and improve heart function.38 The beneficial effects of CPCs in vivo may be due to their partial differentiation, integration into host tissue, and the paracrine functions of the secreted factors such as VEGF and von Willebrand factor (vWF).38 However, the cell survival and long-term retention of CPCs for prolonged therapeutic effects in injured heart remains challenging.39

Environmental Preconditioning of Stem Cells

Hypoxic, oxidative, or heat shock preconditioning

Ischemic tissue environment, oxidative stress, and loss of ECM are the major challenges of cell survival in vivo. Acute ischemia in injured tissues results from the combination of drastic reduction of oxygen tension and the deprivation of nutrients.40 Hromesis defines the brief exposure of tissues to stresses, which can lead to increased recovery after acute stress.40 The beneficial effect of preconditioning was first demonstrated in treating healthy myocardium with intermittent brief ischemia followed by reperfusion, which protected the heart from acute ischemic episodes.41 Since then, various preconditioning strategies include hypoxic preconditioning, exposure to oxidative stress, and heat shock
treatment have been investigated to improve stem cell survival (Table 1). Hypoxia treatment preconditions the cells to adapt to the ischemic environment. Culturing CPCs under hypoxia (2%–5% O₂) in vitro was found to enhance cell survival in a mouse model of myocardial ischemia–reperfusion injury. Moreover, preconditioning ESC-derived NPCs under hypoxia enhanced cell survival with 30%–40% reduction in cell death after transplantation into the ischemic brain of rats, compared to the groups cultivated under normoxia. Similarly, MSCs conditioned under hypoxia promoted angiogenesis and neurogenesis in rat ischemic brain models that mimicked stroke. MSCs exposed to hypoxia in vitro also showed the enhanced survival through the up-regulation of Bcl-2 and Bcl-xL, leading to the reduced infarct size and the enhanced heart functions.

Table 1. Environmental Preconditioning of Stem Cells

| Preconditioning methods | Cell source and pretreatment | Animal model | Performance | Reference |
|-------------------------|------------------------------|--------------|-------------|-----------|
| Hypoxia                 | PSC-derived NPCs exposed to 1% O₂ for 8 h | Rat ischemic brain established through middle cerebral artery occlusion | 30%–40% reduced cell death after transplantation, improved sensorimotor functions compared to normoxic groups | Theus et al. |
|                         | Bone marrow–derived MSCs exposed to 0.5% O₂ for 24 h | Rat brain subjected to middle cerebral artery occlusion (stroke model) | Increased survival and improved brain functional recovery and motor functions compared to normoxic groups | Wei et al. |
|                         | CPCs derived from adult hearts exposed to 0.1% O₂ for 6 h | Mouse heart subjected to coronary ligation (MI model) | Increased survival and heart functions: increased LVS and reduced infarct size | Tang et al. |
|                         | MSCs exposed to 0% O₂ for cyclic short-time periods | Rat heart subjected to coronary occlusion (MI model) | 1.5-fold increase in grafted cell number and improved heart functions: reduced LVDd, LVDs, and infarct size, increased LVS | Wang et al. |
| Exposure to low concentration of oxidative stresses | CPCs derived from adult heart tissue exposed to 100 μM H₂O₂ for 2 days | Rat heart (MI model) | Increased survival and improved heart functions: improved left ventricular cardiac function and reduced scar compared to nonconditioned groups | Pendergrass et al. |
|                         | NPCs exposed to 50 μM H₂O₂ for 24 h | N.A. | Threefold reduced cell death compared to nonconditioned groups | Sharma et al. |
|                         | Wharton Jelly–derived MSCs treated with 200 μM H₂O₂ for 2 h | Mouse heart subjected to left-sided thoracotomy and left anterior descending coronary artery ligation | Reduced myocardial fibrosis, reduced LVDd, LVDs and increased LVS compared to nonconditioned groups | Zhang et al. |
| Heat shock treatment    | hESC-derived cardiomyocytes | Rat heart subjected to thoracotomy and artery ligation | Increased cell engraftment and improved heart functions (increased LVS, reduced LVDd and LVDs) | Laflamme et al. |
|                         | NPCs treated at 43°C for 3 h | N.A. | Heat shock increased HSP-25 expression, which provides protection against apoptosis | Geum et al. |
|                         | Bone marrow MSCs treated at 42°C for 60 min | N.A. | HSP-20 and -70 expression was increased compared to nontreated groups | Moloney et al. |
|                         | CPCs derived from bone marrow 42°C for 3 h | Mouse heart subjected to left-sided thoracotomy and left anterior descending coronary artery ligation | Twofold increase in cell survival, attenuated fibrosis, and improved ischemic heart functions compared to control groups | Feng et al. |

PSC, pluripotent stem cell; NPC, neural progenitor cell; MSC, mesenchymal stem cell; MI, myocardial infarction; CPC, cardiac progenitor cell; LVS, left ventricular shortening; hESC, human embryonic stem cell; LVDd, left ventricular end-diastolic; LVDs, left ventricular end-systolic; HSP, heat shock protein; N.A., not available.
Short-term exposure to low concentration of oxidative stresses in vitro also enhances the survival of stem cells. For instance, a 2-day treatment of CPCs with 100 μM H2O2 in vitro improved the left ventricular cardiac function and reduced cardiac fibrosis in ischemia–reperfusion injury of rat hearts after implantation, as compared to the non-treated groups. Similarly, NPCs exposed to the repeated low doses of H2O2 (up to 50 μM) showed better resistance to acute oxidative stress. The MSCs preconditioned with H2O2 also demonstrated enhanced survival and significant functional improvement of ischemic hearts compared to non-treated groups.

Heat shock treatment also emerges as an attractive approach to increase the cell survival rate. Heat shock preconditioning of NPCs at 43°C for 3 h was reported to reduce cell apoptosis. Similarly, the short-term culture of CPCs at 42°C reduced apoptosis, increased the functionality, and reduced the fibrosis of mouse ischemic myocardium. Transplantation of human ESC-derived cardiomyocytes also used the cells treated by 30–60 min of heat shock at 43°C and improved the formation of functional grafts in a rat model of myocardial ischemia–reperfusion injury, possibly due to the up-regulation of HSPs such as HSP-60, -70, and -90. The exposure of MSCs to high temperature (43°C) was also associated with an increased secretion of HSPs, such as HSP-27 which may contribute to the increased cell survival.

Molecular mechanisms of environmental preconditioning

Antioxidative defense mechanisms in the stem cells mediate their survival from acute ischemic stresses. Examination of the defense mechanisms against the accumulation of reactive oxygen species (ROS) in human iPSCs showed similar mechanisms compared to human ESCs. Preconditioning human PSCs under hypoxia and oxidative stress revealed the induction of anti-apoptotic mechanisms, such as the stabilization of hypoxia-inducible factor (HIF)-2α, which inhibited p53 and increased Bcl-2 expression. MSCs cultured under hypoxia promoted the stabilization of HIF-1α, which is normally degraded by HIF prolyl-4-hydroxylases under normoxia. HIF-1α acts as a regulatory inducer of glycolytic enzymes and an inhibitor of the pyruvate dehydrogenase, which enables the entry into tricarboxylic acid cycle.

The metabolic shift from oxidative phosphorylation to glycolysis under hypoxia reduces ROS generation (Fig. 1). Similarly, a low glucose concentration prevents excessive ROS production. Nutrient deprivation activates mammalian target of rapamycin (mTOR) signaling which also leads to the activation of AKT. VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; TCA cycle, tricarboxylic acid cycle.
generation from stem cells and thus the cells become less prone to oxidative stress–mediated apoptosis.59

Ischemic preconditioning decreases ATP synthesis due to reduced mitochondrial respiration and promotes the opening of mitochondrial ion channels, such as the mitochondrial ATP-sensitive K+ (mitoKATP) channel.60 The mitoKATP opening provokes mild ROS release, which triggers the activation of protein kinase C, leading to the induction of the nuclear factor kappa beta (NFκB) signaling and the synthesis of antioxidant molecules (e.g., MnSOD).60 Consistently, the treatment of MSCs with diazoxide (a mitoKATP opener) to mimic the effects of ischemia-activated NFκB signaling led to the increased survival and expression of trophic factors (e.g., FGF-2).61 Similar treatment of PSC-derived CPCs with diazoxide also promoted cell resistance against oxidative stress.62

Ischemic preconditioning enhances tissue survival after acute ischemia through the activation of the cytoplasmic phosphoinositide 3-kinase (PI3K)/AKT signaling pathway.63 The preconditioning-mediated activation of PI3K/AKT triggers mammalian target of rapamycin (mTOR) and NADPH oxidase (NOX) pathways which slow down the transition from early apoptosis to necrosis or apoptosis in vivo.64 In vitro, the activation of mTOR-AKT-STAT3 signaling in MSCs reduces the secretion of proinflammatory molecules and consequently increases their survival against ischemia (Fig. 1).65 In addition, the activation of PI3K/AKT pathway (e.g., using lysophosphatidic acid, angiopoietin-1) has been reported to promote the secretion of Bcl-2, leading to the enhanced resistance to pro-apoptotic stresses.66

The activation of HSPs through temperature increase or incubation with β-mercaptoethanol promotes the survival of stem cells.6,67 Indeed, HSPs trigger the PI3K/AKT and extracellular signal-regulated kinases (ERK) pathway, which leads to the increased secretion of anti-apoptotic proteins (Fig. 1).68 Importantly, the activation of PI3K/AKT, NFκB, and HSP signaling pathways promotes trophic functions. For instance, enhanced secretion of VEGF, FGF-2, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, and IL-6 has been observed in MSCs upon the activation of PI3K/AKT or NFκB.69 Hence, reducing mitochondrial respiration and activating the PI3K/AKT pathway in vitro can prevent oxidative damage and increase the survival and trophic functions of stem cells in vivo.

Preconditioning Stem Cells as Aggregates

Structural properties of stem cell aggregates

The formation of 3D stem cell aggregates requires both integrin–integrin interactions and homophilic cadherin–cadherin contacts. For instance, N-cadherin expression is required to initiate the spheroid formation from MSCs.16 Similarly, up-regulation of E-cadherin expression was observed in cardiospheres derived from CPCs.70 The formation of neurospheres from NPCs necessitated the expression of neural cell adhesion molecule and N-cadherin.71 Based on the level of cadherin expression, stem cells sort out the aggregate structure according to the differential adhesion hypothesis (Fig. 2). The differential expressions of cell adhesion molecules influence cell surface tension such that cell populations displaying a high expression level of cadherin were found at the interior, whereas cells with high integrin expression were found at the exterior of the aggregates.72 Culturing 3D stem cell aggregates leads to the local gradients of nutrients and oxygen and creates an ischemic-like microenvironment (Fig. 2).73 For instance, MSC spheroids promoted the stabilization of HIF-1α, indicating the formation of a mildly hypoxic environment.74 Similarly, cardiospheres derived from CPCs showed enhanced glycolysis, possibly as a consequence of the formation of hypoxic area.75

**FIG. 2.** Stem cell aggregate formation as a preconditioning treatment. (A) Mechanism of stem cell aggregate formation. Stem cells organize and sort out the structure based on the degree of cadherin expression, according to the differential adhesion hypothesis. (B) Formation of stem cell aggregates promotes the secretion of extracellular matrix (ECM) and trophic factors, as well as creating a mildly hypoxic environment. Stem cell aggregation also could avoid anoikis, promote the expression of antioxidant and anti-apoptotic proteins, and enhance the trophic functions.
Promotion of cell–cell contacts in stem cell aggregates can prevent anoikis due to the secretion of endogenous ECMs.\textsuperscript{76} Cell–cell interactions also increase the expression of gap junction proteins such as connexin-43 which can promote cell survival.\textsuperscript{19} For example, cell sheet derived from cardiomyocytes enhanced cell survival possibly due to the expressions of intercellular adhesion molecules and connexins.\textsuperscript{43,77} Table 2 shows the examples of the secreted endogenous ECM and matrix remodeling proteins in stem cell aggregates,\textsuperscript{78} which can support integrin signaling and mediate cell proliferation and survival. For MSCs, a higher proliferation rate was observed on vitronectin compared to fibronectin, laminin, and collagen type I or type III.\textsuperscript{79} NPCs demonstrated the enhanced proliferation on laminin compared to fibronectin, and endogenous fibronectin was found to protect the cells and induce the proliferation of NPCs after myocardial infarction.\textsuperscript{80,81}

Impediment of oxygen diffusion in 3D aggregates may lead to the formation of an area of hypoxia that promotes the secretion of antioxidants and anti-apoptotic proteins. For instance, proteomics analysis indicated the increased expression of antioxidant proteins such as glutathione S-transferase, chaperones, HSP, and peroxiredoxin in cardiomyocytes in two-dimensional cultures.\textsuperscript{82} Similarly, MSC spheroids enhanced the secretion of Bcl-x\textsubscript{L} and anti-apoptotic proteins.\textsuperscript{83} For instance, proteomics analysis indicated the increased expression of antioxidant proteins such as glutathione S-transferase, chaperones, HSP, and peroxiredoxin in cardiomyocytes in two-dimensional cultures.\textsuperscript{82} Similarly, MSC spheroids enhanced the secretion of Bcl-x\textsubscript{L} and anti-apoptotic proteins.\textsuperscript{83} For instance, proteomics analysis indicated the increased expression of antioxidant proteins such as glutathione S-transferase, chaperones, HSP, and peroxiredoxin in cardiomyocytes in two-dimensional cultures.\textsuperscript{82} Similarly, MSC spheroids enhanced the secretion of Bcl-x\textsubscript{L} and anti-apoptotic proteins.\textsuperscript{83}

Table 2. ENDOGENOUS EXTRACELLULAR MATRIX AND GROWTH FACTOR SECRETION IN STEM OR PROGENITOR CELL AGGREGATES

| Type of stem cell aggregate | Endogenous ECM | Secreted prosurvival factors | Cell source | Reference |
|----------------------------|----------------|-----------------------------|-------------|-----------|
| Cardiosphere               | Matrix remodeling, gene expressions of OL14A1, COL7A1, ITGA2, LAMB1, LAMB3, MMP-3, -10, -11, -13, SELE, PECAM1, SPP1 and collagen type IV | VEGF-A, FGF-2, angiopoietin-2, endothelin, receptor type A, E-selectin, CXCL-1, IL-11, GDF-15, gremlin 1, Fms-like tyrosine kinase 1 (Flt1), B-cell translocation gene 1 | Adult cardiac tissue | Li et al.\textsuperscript{70} Cho et al.\textsuperscript{122} |
| Collagen type I            | ND              | Pluripotent stem cells | Kensah et al.\textsuperscript{78} |
| Mesenchymal stem cell aggregate | Collagens, elastin, tenasin C, fibronectin, and laminin | FGF-2, HGF, and VEGF | Adipose tissue | Amos et al.\textsuperscript{123} Bhang et al.\textsuperscript{74} |
| Fibronectin and laminin   | IL-24, CXCR4, PGE2, TSG-6 | Bone marrow | Wang et al.\textsuperscript{124} Sart et al.\textsuperscript{16} |
| Neurosphere                | Laminin 1, fibronectin | VEGF, PDGFB, TGFA, FGF-5, etc. | Postnatal brain tissues, neural progenitor cells | Campos et al.\textsuperscript{125} Lai et al.\textsuperscript{126} Sart et al.\textsuperscript{127} |
| Fibronectin, laminin, collagen type IV, vitronectin | ND | Embryonic stem cells | |

VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; CXCL-1, chemokine (C-X-C motif) ligand 1; GDF-15, growth differentiation factor 15; Flt1, Fms-like tyrosine kinase 1; PECAM1, platelet endothelial cell adhesion molecule-1; MMP, matrix metalloproteinase; HGF, hepatocyte growth factor; IL, interleukin; CXCR4, chemokine (C-X-C motif) receptor 4; PGE2, prostaglandin E2; TSG-6, tumor necrosis factor-inducible gene 6 protein; PDGFB, platelet-derived growth factor B; TGFA, transforming growth factor A; ND, not determined.
Hydrogels and membranes are usually highly hydrophilic, biocompatible, and nonimmunogenic polymeric materials. Various types of materials have been used as hydrogels for encapsulation: natural polymers, such as Matrigel, collagen, gelatin, agarose, and alginate, or synthetic materials, such as poly(ethylene glycol) (PEG). Solid capsules and hydrogels generally support mass transport of oxygen and nutrients. However, encapsulation can generate the gradients of biomolecules due to diffusion, which may result in the local nutrient and oxygen depletion. Hence, a controlled diffusivity is a critical parameter to promote the encapsulated stem cell survival, which can be achieved by controlling the polymer concentration.

**Encapsulation prevents anoikis and regulates trophic functions**

Adhesive hydrogels (i.e., containing or binding ECM proteins) provide 3D substrates for stem cell anchorage and facilitate ECM remodeling, hence limiting the anoikis and promoting cell migration. For instance, collagen or gelatin hydrogels containing RGD sequence and matrix metalloproteinase (MMP) binding sites supported efficient propagation, survival, and migration of stem cells. Recent developments of functionalization and bioconjugation methods for nonadhesive hydrogels, such as PEG and hyaluronic acid (HA), with integrin- and MMP-binding domains provide biochemically and biomechanically controlled semisolid scaffolds to regulate cellular behaviors. For instance, the controlled distribution of RGD sequences on HA-based hydrogels regulated integrin-expression profile, cell shape, proliferation, and survival of MSCs. In addition, modulation of the stiffness of alginate or PEG-silica gel functionalized with RGD regulated NPC survival and expansion. Moreover, the ECM pattern and stiffness in hydrogels affect trophic functions of stem or progenitor cells. For examples, an increased density of RGD sequence on gellan gum hydrogels promoted the secretion of neurotrophic factors from encapsulated MSCs. Cross-linked methacrylate-HA hydrogels with low modulus (1.5 and 2.6 kPa) enhanced the secretion of angiogenic factors from encapsulated stem cells compared to stiffer hydrogels (3.8 and 7.4 kPa). Consequently, modulation of hydrogel properties can provide cell delivery vehicles which can enhance the therapeutic functions of stem or progenitor cells in vivo.

Alternatively, nonadhesive hydrogels (such as agarose and PEG) surrounding stem cells in suspension promote the formation and the confinement of stem cell aggregates with controlled size. As demonstrated in PSCs, the aggregate size affected cell survival and the lineage commitment due to

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**Table 3. Hydrogel-Based Methods for Cell Transplantation**

| Hydrogel type | Cell source | Animal model | Performance | Reference |
|---------------|-------------|--------------|-------------|-----------|
| Agarose       | CPCs derived from adult heart tissues | Mouse heart subjected to artery ligation (MI model) | Fivefold increase survival and improved heart functions: twofold reduced infarct size, 10% increase left ventricular ejection fraction compared to non-encapsulated groups | Mayfield et al.¹⁰⁴ |
| PGE2-functionalized biodegradable hydrogel | MSCs derived from bone marrow | Rat heart subjected to coronary artery ligation (MI model) | Increased survival decreased the number of CD8⁺ T cells. Improved heart functions: increased fractional shortening, reduced scar size and reduced LVDs and LVDd compared to non-encapsulated groups | Dhingra et al.¹⁰⁶ |
| Gelatin/laminin | Warthon Jelly–derived MSCs | Rat brain subjected to ouabain-mediating excitotoxicity | Improved cell survival post-transplantation, decreased activated microglia/macrophages compared to non-encapsulated groups | Sarnowska et al.¹⁰⁷ |
| Hyaluronic acid/gelatin/PEGDA | Immortalized NPCs from fetal tissues | Rat brain striatum; rat spinal cord subjected to laminectomy | Increased cell survival, reduced host immune response compared to non-encapsulated groups | Liang et al.¹⁰⁸ |
| Hyaluronan/heparin sulfate/collagen | Adult tissue and embryonic stem cell-derived NPCs | Mouse brain subjected to cortical photothermotic stroke | Twofold increase in cell survival, significant reduction of microglia/macrophage infiltration compared to non-encapsulated groups | Zhong et al.¹⁰³ |

PEGDA, poly(ethylene glycol) diacrylate; LVDd, left ventricular end-diastolic; LVDs, left ventricular end-systolic.
diffusion limitation and the size-dependent ratio of outer endoderm cells and inner undifferentiated cells in EBs.\textsuperscript{96,97} In addition, the degree of spatial confinement may affect local accumulation of paracrine or autocrine factors to regulate the phenotype of human PSC aggregates.\textsuperscript{98} While further investigations are required to determine the role of compression or confinement on encapsulated stem cell aggregates, the up-regulation of secreted growth factors (e.g., transforming growth factor beta, bone morphogenetic protein) has been observed in MSCs encapsulated in nonadhesive hydrogels with compression force.\textsuperscript{99}

Encapsulation provides immune isolation and enhances cell survival

Stem cells encapsulated in core-shell capsules or embedded within hydrogels demonstrate the limited inflammation and activation of immune responses. For instance, reduced IgG binding to EBs embedded in core-shell alginate-chitosan-alginate capsules was observed compared to non-encapsulated cells.\textsuperscript{100} Moreover, functionalization of PEG hydrogels with tumor necrosis factor $\alpha$-antagonizing molecules (e.g., WP9QY peptide) further reduced the immune response.\textsuperscript{101} CPCs encapsulated in alginate hydrogels that were functionalized with superoxide dismutases demonstrated the enhanced resistance against oxidative stress.\textsuperscript{102} When encapsulated within cross-linked hyaluronan/heparin/collagen hydrogels, NPCs showed the significantly reduced immune rejection and macrophage infiltration into the infarct zone in a mouse stroke model.\textsuperscript{103}

Besides reducing immune response, hydrogel encapsulation enhances cell survival and functions \textit{in vivo}. For example, encapsulation of CPCs in agarose hydrogels improved left ventricular functions in mouse heart subjected to MI.\textsuperscript{104} The MSCs encapsulated in alginate also retained high viability and trophic functions (e.g., secretion of VEGF, IGF-1, FGF-2, and HGF), while minimizing the immune response once injected into hearts.\textsuperscript{105} The injection of encapsulated MSCs in hydrogel functionalized with PGE2 in an MI model of rat heart reduced scar area and improved left ventricular functions.\textsuperscript{106} Encapsulated MSCs in gelatin/laminin gels enhanced survival while reducing the neuro-inflammation in ischemic rat hippocampus.\textsuperscript{107} Similarly, the encapsulation of NPCs in HA/gelatin/PEG diacrylate sustained cell survival after implantation in mouse striatum.\textsuperscript{108} By modulating the biochemical and biomechanical properties of hydrogels, the trophic function of stem cells and their \textit{in vivo} retention can be enhanced.

**Cell Dose Preparation for \textit{In Vivo} Study**

For the safe and efficient transplantation of preconditioned stem cells in injured sites, accurate characterization of cells and hydrogels with minimal invasiveness are required. The parameters of cell dose preparation include cell concentration, volume, and buffer formulation. Also, the cell delivery methods impact the cell viability postinjection, where injectable hydrogels may protect the cells from the extensional flow during injection.\textsuperscript{109} These parameters have to be optimized based on specific cell types and animal models.

**Cell dose preparation**

The environmental conditioning of stem cells with ischemic, hypoxic, and oxidative treatments could be performed before cell harvesting, while the cells need additional steps for aggregate formation or formulation in hydrogels after

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**FIG. 3.** Stem cell encapsulation as a preconditioning treatment. (A) Liquid core/solid shell encapsulation promotes aggregate formation and provides mass transport of biomolecules and immune isolation. (B) Stem cell encapsulation in nonadhesive hydrogels promotes aggregate formation and provides mass transport of biomolecules and immune isolation. (C) Stem cell encapsulation in adhesive hydrogels (i.e., containing integrin- and MMP-binding sites) promotes stem cell adhesion and provides mass transport of biomolecules and immune isolation.
the harvesting. For high dose study (~10^7–10^8 cells per dose) such as in heart diseases, the cells are highly concentrated compared to the low dose (~10^5–10^6 cells per dose) that is usually used in neural diseases. For the dose formulation, prosurvival factors may also be added to enhance cell survival in addition to hydrogels, such as Bcl-xL peptide to block mitochondrial death pathways, IGF-1 to activate cytoprotective AKT pathways, and the caspase inhibitor ZvAD-fmk to reduce apoptosis. The accurate characterizations of biochemical, biomechanical, immunological, and diffusion properties of the prosurvival factors and hydrogels have to be provided. For example, it has been shown that mechanical properties of alginate gel contributed to cell protection rather than the biochemical properties of alginate biopolymers. PEG-based microcryogels with predefined size and shape have also been shown to augment the paracrine functions and the tissue integration as injectable cell delivery vehicles due to the cell protection from shear-induced damage and the controlled cell localization.

To ensure proper stem cell retention and function after transplantation, the controlled functional properties of stem cells in vitro in terms of trophic factor secretion, immunogenicity, and the expressions of antioxidants and anti-apoptotic proteins may need to be performed. The control of stem cell aggregate size, which regulates stem cell properties and viability, has to be rigorously achieved as well. The specifications that are used for quality control are mainly established based on the experimental results. In this regard, the in vitro measurements that can correlate with the in vivo behaviors are critical, yet challenging.

Cell dose delivery methods

Stem cells are usually delivered via injection through systemic infusion (i.e., intravenous or intra-arterial) or local transplantation at the injured sites. More tissue-specific routes are also used such as intraventricular delivery for neural tissue and transendocardial delivery for cardiovascular diseases. These different cell dose delivery routes can affect the transplantation outcome. For example, transendocardial route showed higher cell retention and increased vascularity than intracoronary delivery. Transplantation of MSCs through endocardium showed enhanced cell retention and improved left ventricular ejection fraction compared to intravascular infusion. For stroke, stem cell delivery through the intracerebral route was reported as the most invasive method. Alternatively, intracisternal/cerebroventricular and intravascular delivery routes showed less invasiveness, but cell migration to the injured site was limited. Similarly, for the treatment of spinal cord injury, intraleision delivery displayed enhanced cell retention and facilitated secretion of neurotrophic factors while intravascular infusion had limited cell engraftment, which reduced the therapeutic functions.

The cell delivery methods affect cell viability and the actual dose administered due to flow stress, backpressure, tissue density, and so forth. To reduce the variations in manual injection, automated methods (e.g., computer-controlled syringe pump) have also been developed that show similar outcomes for cell viability with the controlled cell volume and flow rate. Among various parameters, length and diameter of the tubing as well as cell density have been shown to be critical factors for better survival and retention of therapeutic cells. The automated methods may be used when systemic delivery of single cells is required. For example, single cells of MSCs can transmigrate through the endothelial barrier via paracellular or transcellular diapedesis.

For large cellular constructs such as stem cell aggregates or encapsulated cells, the cell delivery methods still need to be better designed to control the cell dose and maintain the structure integrity.

Conclusions

Stem cell preconditioning through chronic environmental stresses, aggregate formation, and hydrogel encapsulation improves cell survival and biological functions of transplanted cells in vivo. The selection of preconditioning strategy must consider the cell delivery route and the specific clinical application. For example, intravenous or intra-arterial injections are incompatible with the transplantation of encapsulated cells or aggregates. In these cases, environmental preconditioning, which enables the injection of single cell suspension, is more suitable for cell survival and function. In contrast, the local stem cell injection to the injury site can enhance stem cell retention in situ. Thus, stem cell preconditioning as aggregates and/or encapsulated cells within hydrogels is more suitable for cell survival and maintaining biological activity and for restoring the damaged tissue’s integrity. However, the molecular mechanisms of environmental preconditioning need to be further elucidated. Further investigations are also required to understand the structural and biological properties of stem cell aggregates in order to enhance cell survival and trophic functions. In addition, the influence of biochemical and biophysical properties of 3D hydrogels on stem cell behaviors merits further study towards the increased functionality in vivo. Preparation of cell dose needs to be carefully designed based on cell types, animal models, and the delivery methods. Taken together, stem cell preconditioning can be applied prior to transplantation towards the safe long-term efficacy of stem cell–based therapy.

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Author Disclosure Statement

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Abbreviations Used
3D = three-dimensional
BDNF = brain-derived neurotrophic factor
CPC = cardiac progenitor cell
EB = embryoid body
ECM = extracellular matrix
ERK = extracellular signal-regulated kinases
ESC = embryonic stem cell
FGF = fibroblast growth factor
HA = hyaluronic acid
HGF = hepatocyte growth factor
HIP = hypoxia-inducible factor
HSP = heat shock protein
IGF = insulin-like growth factor
IL = interleukin
iPSC = induced pluripotent stem cell
MI = myocardial infarction
MMIP = matrix metalloproteinase
MSC = mesenchymal stem cell
mTOR = mammalian target of rapamycin
NFkB = nuclear factor kappa beta
NPC = neural progenitor cell
PEG = poly(ethylene glycol)
PGE2 = prostaglandin E2
P3K = phosphoinositide 3-kinase
PSC = pluripotent stem cell
ROS = reactive oxygen species
VEGF = vascular endothelial growth factor
vWF = von Willebrand factor