Stem Cell Therapy in Brain Ischemia: The Role of Mitochondrial Transfer

Lei Huang,^{1,2} Cesar Reis,^{3,4} Warren W. Boling,¹ and John H. Zhang^{1,2,5}

Mitochondrial dysfunction is an important pathological process in the setting of ischemic brain injury. Stem cell-mediated mitochondrial transfer provides an efficient intercellular process to supply additional mitochondria in the ischemic brain tissues. In this review, we summarize the mitochondrial pathology associated with brain ischemia, mechanisms of stem cell-mediated mitochondrial transfer, and in vitro/in vivo experimental findings of mitochondrial transfer from stem cells to ischemic vascular endothelial cells/neurons as potential therapeutic strategy in the management of ischemic brain injury.

Keywords: MSCs, mitochondrial dysfunction, mitochondrial transfer, Miro1, TNTs, Cx43, brain ischemia

Introduction

CELL-BASED STEM CELL therapy for ischemic brain injury has been approved for the safety and efficacy in animal models and some clinical studies. The identified mechanisms of neuroprotection consist of (1) the direct replacement of injured neurons through differentiation [1,2] and (2) the paracrine effects through secreting a broad spectrum of neurotrophic factors, cytokines, and chemokines in favor of the regeneration and suppressing neuroinflammation. Recently, emerging evidence demonstrates the novel component-based mechanism that stem cells are capable of transferring the mitochondria to ischemic neuron [3,4].

Mitochondrial dysfunction leads to cellular damage and apoptosis [5,6]. Thus, a rescue mechanism that enables mitochondrial transfer ensures a promising therapeutic/regeneration potential [3,4,7,8]. Stem cell-mediated mitochondrial donations provide an efficient intercellular process that supplies additional mitochondria in diseased cells/tissue [4,8–12]. Mitochondria transferred from stem cells to injured cells are capable of recovering their cellular metabolism capabilities by meeting the high oxidative energy demands.

Thus, mitochondrial transfer would be efficient to rescue damaged cells in crisis, compared with other relatively time-consuming approaches that promote mitochondrial biosynthesis. In addition to the neuroprotective approaches identified in the animal models of ischemic stroke [13–16], mitochondrial transfer reveals a new neuroprotective mechanism of stem cell therapy against ischemic stroke.

In this review, we summarize the mitochondrial pathology associated with brain ischemia and the mechanisms of stem cell-mediated mitochondrial transfer. The in vitro/

in vivo experimental findings of mitochondrial transfer from stem cells to ischemic vascular endothelial cells/neurons provide an update on the use of stem cell therapy in the setting of brain ischemia.

Mitochondrial dysfunctions after brain ischemia

Mitochondria, a DNA containing cellular organelle, plays an important role in producing adenosine triphosphate (ATP), maintaining redox balance, and regulating cellular apoptosis [6,17]. After brain ischemia, mitochondrial homeostasis is impaired in neurons [18], which constitutes a comprehensive of pathological process (Fig. 1).

Mitochondrial permeability transition pore (MPTP) is open subsequent to the ischemia-induced matrix Ca²⁺ overload and the elevated oxidative stress [19–21]. The opening of MPTP leads to increased mitochondrial permeability, allowing solutes such as water, large molecules, and ions to enter freely into the mitochondrial matrix. This increase in mitochondrial permeability causes mitochondrial swelling and rupture of the outer membrane and impairment of the electron transport chain, resulting in a large release of reactive oxygen species (ROS) [22,23].

Furthermore, elevated mitochondrial permeability also causes a decrease in membrane potential and further exacerbates the reduction in cellular mitochondrial ATP levels and the elevation of intracellular Ca²⁺ concentration [24]. Consequently, endogenous apoptotic pathways are activated, resulting in neuronal damage [25]. Upon the onset of blood reperfusion, a substantial increase in mitochondrial ROS due to the long-lasting MPTP opening is followed by irreversible alteration in cellular biogenesis [26]. The

Departments of ¹Neurosurgery, ²Physiology and Pharmacology, ⁴Preventive Medicine, and ⁵Anesthesiology, Loma Linda University, Loma Linda, California, USA.

³Occupational Medicine, Southern California Kaiser Permanente, Riverside, California, USA.

FIG. 1. Mitochondrial pathophysiology in brain ischemia. **(A)** The function and structure of healthy mitochondria; **(B)** the pathological process of mitochondria subjected to ischemia. Color images are available online.

preservation of mitochondrial function is important to rescue ischemic neurons from oxidative stress and the subsequent cell death process.

The capability of stem cells to transfer mitochondria

Stem cell-mediated mitochondria are quite an intriguing phenomenon. Spees et al. first observed this process in a coculture system where mitochondrial DNA (mtDNA) of donor human mesenchymal stem cells (MSCs) was found in recipient cells devoid of mitochondria, and such mitochondrial rescue promoted the functional recovery of recipient cells [12]. However, the exact route of stem cell-mediated mitochondrial transfer could not be differentiated between a direct route through an intercellular structure such as tunneling nanotubes (TNTs) and through the secretion of mitochondria containing vesicles [12]. This study was followed by a plethora of studies that validated the capability of mitochondrial transfer between stem cells and the different types of damaged cells [27–32]. These additional mitochondria are transferred from stem cells to damaged cells with dysfunctional mitochondria, thereby improving the impaired cellular metabolism.

The different types of stem cell such as bone marrow-derived MSCs (BM-MSCs), endothelial progenitor cells (EPCs), adipose-tissue-derived stem cells, induced pluripotent stem cells (iPSCs), and Wharton's jelly-derived MSCs were all able to donate mitochondria to various damaged cells in favor of rescuing cellular bioenergetics [3,4,9,10,27–29,31–36]. After coculture with stem cells, mitochondrial respiration and ATP levels upregulated along with a reduction of oxidative damage in injured cells or tissues [4,8–10,30,33,37].

Signals triggering mitochondria transfer from stem cells

The local microenvironment cues released from the injured recipient cells most likely trigger the transfer signaling

in stem cells [38]. In the context of major cellular stress, intracellular molecules called damage-associated molecular patterns (DAMPs) are released into the extracellular space and act as the stress signal for the body [39]. Mitochondrial DAMPs include damaged mitochondria, mtDNA, and mitochondrial products released by injured cells [40]. A bidirectional mitochondrial communication through the coexisting microenvironment was observed between MSCs and stressed recipient cells [38]. Mahrouf-Yorgov et al. found cells under stress underwent mitochondrial dysfunction and released mtDNA. After mtDNA were engulfed by cocultured MSCs, cytoprotective enzyme heme oxygenase-1 expression increased a process that potentially enhanced mitochondrial donation capabilities of MSCs [38].

Cells under oxidative stress and inflammation also release ROS, which may facilitate the cross talk between injured cells and MSCs. ROS scavengers abolished the mtDNA transfer from injured cells to MSCs and thereby the repair function of MSCs [38]. A previous in vitro study showed that cytochrome c released from cerebellar granule cells undergoing apoptosis or excitotoxicity enhanced the extracellular export of isolated mitochondria [41]. The subsequent caspase-3 activation in the early stages of apoptosis triggered mitochondria donation from MSCs to rescue ultraviolet-damaged PC12 cells [42].

High intracellular cytokines such as tumor necrosis factor alpha (TNF α) and the activation of NF- κ B signaling in degenerated retinal ganglion cells also triggered the mitochondria transfer from iPSC-MSCs [31]. After exposed to cisplatin, the translocation of p53 to the mitochondria of neurotoxic neural stem cells conferred a danger signal to prompt the mitochondrial transfer from MSCs [43]. An intriguing question remains as to the threshold of cellular damage that initiates the intercellular transfer of functional mitochondria.

In addition to the signaling from injured cells, the intrinsic signaling in stem cells may help the mitochondrial transfer. Recent findings showed the astrocytic release of extracellular mitochondrial particles was mediated by a calcium-dependent

mechanism involving *CD38*/cyclic adenosine di-phosphate (ADP) ribose signaling in the astrocytes [7]. *CD38* catalyzed synthesis of the calcium messenger cyclic ADP ribose in mitochondrial membranes, which further increased mitochondrial donation in a calcium-dependent manner. Although *CD38* siRNA reduced extracellular mitochondrial transfer from astrocytes by suppressing *CD38* signaling, the activation of astrocytic *CD38* signaling increased the extracellular release of mitochondria from astrocytes [7]. This finding suggests that the healthy cells can detect a certain degree of metabolic disturbance in the adjacent stressed cell and help restore their function, therefore preventing the initiation of cell apoptosis [44].

Routes and mechanisms of intercellular mitochondria delivery from stem cells

Various signaling molecules have been shown to participate in mitochondrial transfer from MSCs. The routes of mitochondrial transfer from stem cells to injured cells involved the formation of intercellular TNTs or gap junctions, the extracellular secretion of microvesicles (MVs) containing mitochondria, and the process of cell fusion. Although TNTs or gap junctions sustain the communication between physically connected cells through membrane tubular structures, extracellular vesicles allow the transfer of information between separated cells, ensuring communication over long distances [45]. Cell fusion refers to a cellular process that the individual cells combine to form multinuclear cells by fusing their membranes and sharing organelles and cytosolic compounds [44].

A plethora of studies demonstrate mitochondrial transfer through the formation of TNT between MSCs and cells that are damaged. Motor-adaptor protein complexes regulate mitochondrial transport and homeostasis [46]. Miro 1 and Miro 2, two domains related to Rho-GTPases that interact with other accessory proteins, facilitating the migration of mitochondria through cytoplasmic tunnel tube extensions between two cells. In a coculture system of BM-MSCs and alveolar epithelial cells, Miro1 regulated intercellular mitochondria transfer through nanotube transportation. Miro1 overexpression enhanced the rescue potential and epithelial cell repair of MSCs [37]. In the setting of anthracycline-induced cardiomyopathy, higher expressions of Miro 1 in iPSCs resulted in higher efficacy of mitochondrial transfer than that of BM-MSCs. Instead, Miro1 knockdown with short hairpin RNA reduced the efficacy of mitochondrial donation [36]. In addition, when cells were treated with cytochalasin B, leading to inhibition of actin polymerization and tunnel tube formation, the mitochondrial transfer was absent [36].

Early studies highlight the involvement of $\text{TNF}\alpha$ -induced protein 2 (*TNF\alpha ip2*), a 73-kDa cytosolic protein, in inducing the membrane protrusion of TNTs formation during coculture of immune cells and HEK293T cells [47]. In response to $\text{TNF}\alpha$, *TNF-\alpha/NF-\kappa B/TNF\alpha ip2* signaling pathway was upregulated, and iPSCs further promoted TNT formation [36]. In the study of astrocytic mitochondrial donation, the tumor suppressor molecule p53 and *Akt/PI3K/mTOR* signaling pathway have been shown to play a role in promoting TNT formation [48]. Recent pioneer studies demonstrated that connexin 43 (Cx43) mediated intercellular communication through TNTs [49]. Cx43 overexpression in iPSC-

MSCs facilitated the mitochondrial transfer between iPSC-MSCs and epithelial cells by the upregulation of TNT formations [50]. Cx43-based intercellular gap junctional communication also occurred in coculture of MSCs and endothelial cells [51].

Stem cells can package mitochondria in MVs and transport them to other cells. In MSC cytoplasm, mitochondria were packaged into autophagy marker light chain 3-containing vesicles that were integrated into outward budding blebs in the plasma membrane [52]. The mitochondria containing MVs were secreted from BM-MSCs and subsequently engulfed by the epithelium cell, leading to improved alveolar bioenergetics [29]. This MV-dependent mitochondrial transfer was also reported between astrocytes and injured neurons [7]. In addition to its regulation of TNT formation, Cx43 has been shown to mediate intercellular talk through extracellular vesicles [53]. In a Ca^{2+} -dependent manner, Cx43 regulated intercellular mitochondrial transfer from BM-MSCs to LPS-injured alveolar epithelial cells by nanotube formation and MVs [29]. These findings support Cx43-dependent mechanisms in viable mitochondrial transfer.

The partial or total cell fusion process can occur between stem cells and cardiomyocytes, leading to mitochondrial functional restoration and cardiac regeneration in the setting of stem cell therapy for myocardial infarction [54]. In addition to direct differentiation, human MSCs are able to fuse with heat-shocked small airway epithelial cells during ex vivo repair [55].

Stem cell-mediated mitochondria transfer in ischemic brain injury

Babenko et al. first reported an improved therapeutic potency of primed MSCs by precoculture with rat cortical neurons [3]. When coculturing MSCs with cortical neurons, a bidirectional transfer of cellular contents was reported through intracellular contact. While cytosol fluorescent probes were preferentially transferred from neurons toward MSCs, the presence of a reversed transfer of mitochondria from MSCs to cortical neurons was also observed [3]. In the absence of differentiation induction, precoculture with neurons enabled MSCs to produce slightly more brain-derived neurotrophic factors with intracellular redistribution. Intravenous injections of those postcocultivated MSCs in the rat model of middle cerebral artery occlusion (MCAO) resulted in a better neurological function compared with that of unprimed MSCs [3].

After discovering mitochondrial transfer from healthy astrocytic cells to ischemic neuronal cells [7], Hayakawa et al. consistently demonstrated successful mitochondrial transfer from EPCs into ischemic brain endothelial cells [9]. Using an in vitro oxygen-glucose deprivation (OGD) stroke model, they consistently proved the protective effect of EPC-derived mitochondria. The transfer of EPC-derived extracellular mitochondria increased transcellular endothelial permeability, mitochondrial biogenesis, mtDNA copy number, and intracellular ATP levels, thus enhancing the endothelial tightness possible through upregulating specific genes for protection [9].

Borlongan et al. further confirmed stem cell therapy attenuated experimental stroke deficits in cultured neuronal cells by restoring mitochondrial function [4]. The finding in OGD model in vitro was translated to the rat model of focal

brain ischemia in vivo. The oxidative phosphorylation capacity of mitochondria was rescued in stroke animals transplanted with EPCs. Confocal microscopy provided the direct evidence of mitochondrial transfer from EPCs to ischemic neurons, leading to improved neurobehavioral deficits and brain tissue histopathology [4].

Liu et al. reported that MSCs rescued ischemic endothelial cells by transferring mitochondria in an in vitro ischemia–reperfusion model [10]. Through a TNT-like structure, the frequent and almost unidirectional mitochondrial transfer from MSCs to injured endothelial cells rescued aerobic respiration and protected endothelial cells against apoptosis [10]. The TNT-like structure formation may be triggered by a defense and rescue mechanism involving in surface exposure phosphatidylserines on the apoptotic endothelial cells and stem cell recognition [10]. A follow-up in vivo study using a rat model of MCAO demonstrated that the injured cerebral microvasculature accepted the mitochondria transferred from transplanted stem cells, thereby significantly improving mitochondrial activity of injured microvasculature, enhancing angiogenesis, reducing infarct volume, and improving functional recovery [8].

These studies demonstrate the capabilities of mitochondrial transfer from astrocytes and stem cells (EPCs or MSCs) to ischemic neurons or endothelial cells. The results include renewing cellular bioenergetics and/or providing functional benefits. Borlongan et al. stressed that an exogenous stem cell-based mitochondria approach would have superior efficacy over transient and insufficient mitochondrial transfer from endogenous astrocytes after ischemic stroke [5].

Indeed, astrocytes or stem cells could potentially serve as donor cells for mitochondria isolation. The transplantation of isolated xenogeneic mitochondria has shown to reduce the infarction size and improve behavioral outcomes in a rat model of ischemic stroke. Intact respiratory activity was essential for the neuroprotective efficacy [56]. Preclinical

studies showed that transplantation of autologous mitochondria protected the heart from ischemic-reperfusion injury [57]. Preclinical experiments will help elucidate the therapeutic potency by directly comparing the effects of stem cell-mediated mitochondrial transfer with transplantation of isolated mitochondria in the treatment of ischemic brain injury.

Conclusion and future directions

Mitochondrial dysfunction plays a vital role in ischemic brain injury. In vitro and in vivo preclinical studies have demonstrated how mitochondrial transfer to ischemic vascular endothelial cells/neurons contributed to the treatment and regenerative effects of stem cells. The stem cell-mediated mitochondrial transfer reveals the therapeutic potential of stem cells to restore bioenergetics in ischemic cells.

Preprimed stem cells may potentially enhance mitochondrial transfer capabilities. Triggered by damage signaling released from injured cells, mitochondrial transfer is an active process involving the formation of TNTs/gap junction, vesicular transfer, or cell fusion. Miro1-regulated TNTs formation, Cx43-regulated gap junctions/TNTs formation, and release of extracellular MVs and cell fusion are current routes of stem cell-mediated mitochondrial transfer (Fig. 2). The activation of *TNF- α /NF- κ B/TNF α ip2*, calcium-dependent *CD38/cyclic ADP ribose*, and *p53/Akt/PI3K/mTOR* signaling may facilitate mitochondrial transfer. However, the choice of signaling pathways and other downstream signals have not yet been determined.

It should be noted that stem cells not only serve as mitochondrial donors, but they also exert paracrine effects. The relative roles of mitochondrial transfer versus paracrine secretion in neuroprotective effects provided by stem cell therapy are not clear. The mitochondrial transfer and paracrine factors of MSCs have been shown to be independent mechanisms to protect against stressed cardiomyocytes [36].

FIG. 2. Mechanism of MSCs mediated mitochondrial transfer. In ischemic cell, multiple damage signaling associated with mitochondrial dysfunction trigger the mitochondrial donation of MSCs. Subsequently in MSCs, active processes occur, including the Miro1-regulated TNTs formation, Cx43-regulated gap junctions/TNTs formation, release of extracellular MVs and cell fusion, which enable to transfer the additional mitochondria to ischemic cell. Cx 43, connexin 43; *HO-1*, heme oxygenases-1; MSCs, mesenchymal stem cells; mtDNA, mitochondria DNA; MVs, microvesicles; ROS, reactive free radical; *TNF α* , tumor necrosis factor alpha; *TNF α ip2*, TNF α -induced protein 2; TNT, tunneling nanotubes. Color images are available online.

Although BM-MSCs may decrease their secretion capacity after mitochondrial donation to attached alveolus type 2 cells, nonattached mitochondria-competent BM-MSCs could continue to secrete protective paracrine effects [29].

Given that the neuroprotective mechanisms of stem cells are not limited to mitochondrial transfer only, they may provide improved overall treatment efficacy compared with the transplantation of isolated mitochondria in the setting of ischemic stroke. Stem cell-mediated mitochondrial transfer may open a new avenue of cell-based stem cell research for ischemic stroke. Stem cells could potentially donate other organelles such as lysosomes, exosomes, and endosomes to ischemic cells, favoring further functional repair of ischemic neurons. Future studies are needed to clarify these unanswered questions to fully optimize the therapeutic regime of stem cell therapy in the management of patients with ischemic brain injury.

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Address correspondence to:

Lei Huang, MD

Department of Neurosurgery

School of Medicine, Loma Linda University

Risley Hall, Room 213

11041 Campus Street

Loma Linda, CA 92354

USA

E-mail: lhuang@llu.edu

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