

Healthy mitochondria for stroke cells

Eleonora Russo, Eleonora Napoli¹, Cesar V. Borlongan

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Abstract:

Stroke is a debilitating disease that remains as a significant unmet need. Although our understanding of the disease pathology has advanced over the years, treatment options for stroke are limited. Recent studies have implicated the important role of healthy mitochondria in neuroprotection against stroke. Under the stroke pathological condition, transfer of healthy mitochondria is observed from astrocytes to ischemic neurons. However, without additional therapeutic intervention, such astrocyte-to-neuron transfer of mitochondria may not sufficiently afford a robust and stable therapeutic effect against the devastating primary insult and progressive neurodegeneration associated with stroke. We now explore the concept that transplantation of exogenous stem cells may serve as efficacious sources of healthy mitochondria for ischemic cells, not only neurons but also endothelial cells. This review captures the recent advances on the therapeutic potential of mitochondrial transfer as a novel stroke treatment. This paper is a review article. Referred literature in this article has been listed in the references section. The data sets supporting the conclusions of this article are available online by searching various databases, including PubMed.

Keywords:

Astrocytes, endothelial cells, mitochondria, neurons, stem cells, stroke

Introduction

Stroke is a leading cause of death worldwide and still remains a serious determinant of severe disability.^[1] Despite our growing knowledge of stroke pathology, only one Food and Drugs Administration (FDA) approved pharmacological therapy is available for the treatment of stroke, tissue-type plasminogen activator (tPA).^[2] However, the narrow therapeutic window of tPA, displaying limited efficacy within 4.5 hours after stroke onset, and the secondary hemorrhagic risk restrict the number of patients that can benefit from it.^[2] The extensive evidences of the crucial role of mitochondria in ischemic cascade injury and the recent findings of mitochondria transfer provide a strategic basis for a new stem cell-based therapeutic strategy involving the transfer of healthy mitochondria into stroke cells.^[3] This review explores the

potential benefit of stem cells as pivotal sources of healthy mitochondria not only for ischemic neurons but also for ischemic endothelial cells, advancing the application of mitochondria transfer for the treatment of ischemic stroke.

Stroke and Mitochondria

Ischemic stroke is the second leading cause of death worldwide behind heart diseases, and it accounts for the 87% of stroke cases in the United States.^[1] Ischemic stroke results in a blood flow reduction in the brain tissue supplied by the occluded artery.^[2] A direct consequence of oxygen and glucose deprivation (OGD) during stroke is the dysfunction of mitochondria that impair oxidative metabolism and contributes to neuronal death and inflammation.^[3] In particular, the mitochondrial impairment after stroke results in reduction of adenosine triphosphate (ATP) production because mitochondria are responsible for the 92% of the total ATP production of the cell.^[3,4] In addition, the mitochondrial dysfunction

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Center of Excellence
for Aging and Brain
Repair, Department
of Neurosurgery and
Brain Repair, College of
Medicine, University of
South Florida, Morsani
College of Medicine,
Tampa, Florida,

¹Department of Molecular
Biosciences, School of
Veterinary Medicine,
University of California,
Davis, California, USA

Address for correspondence:

Dr. Cesar V. Borlongan,
College of Medicine,
University of South
Florida Morsani, Tampa
33612, Florida, USA.
E-mail: cborlong@health.usf.edu

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induces excessive production of reactive oxidative species (ROS), and consequently reactive nitrogen species (RNS), especially during the reperfusion in which the level of oxygen is restored.^[3] Both ROS and RNS cause oxidative stress that triggers the direct damage of proteins, lipids, and deoxyribonucleic acid (DNA).^[3] However, mitochondria exert different hierarchical quality control mechanisms against oxidative stress including mitochondrial fusion and fission to protect mitochondria against stress and damage, ensuring the selective removal of dysfunctional mitochondria by mitophagy.^[3] Finally, excessively damaged mitochondria are characterized by an increase of membrane permeability that allows the release of proapoptotic molecules in the cytoplasm triggering apoptotic cell death.^[3] Therefore, mitochondria dysfunction has a central role in stroke injury. Many studies have advanced the potential of regenerative medicine, specifically stem cell therapy, for stroke. The therapeutic potential of stem cells is due to cell replacement, secretion of growth factors, and anti-inflammatory molecules as well as promoting endogenous repair mechanisms facilitating the migration of endogenous neural stem cells from the neurogenic niche to the lesion site.^[5] To this end, we entertain the idea of stem cell-based mitochondria transfer for stroke.

Transfer of Healthy Mitochondria after Stroke

Recently, it has been demonstrated that a novel beneficial mechanism of stem cells involves transferring healthy mitochondria into damaged cells.^[6,7] Mitochondria can be released through tunneling nanotubes or microvesicles and uptake by recipient cells.^[6,7] Mitochondria transfer can also occur through gap junctions, cell fusion, and direct uptake of isolated mitochondria.^[6,7] To date, we do not know yet what condition that predisposes a cell to release mitochondria as well as which cellular or molecular signals that can induce these organelles to be released from donor cell to another cell, but such transfer has been documented to occur during pathological conditions which can help damaged cells to recover their functions.^[6,7] In the last few years, mitochondrial transfer between different cell types has been demonstrated in several cell types including mesenchymal stem cells and pulmonary alveoli, astrocytes and neurons, and bone marrow–mesenchymal stem cells (BM-MS).^[8-10]

The transfer of healthy mitochondria into damaged cells is viewed as a novel therapeutic strategy for stroke.^[6] Dr. Lo *et al.* focused their attention on the endothelial progenitor cells (EPCs) that represent a subset of immature endothelial cells circulating in the bloodstream.^[11] The EPCs can migrate and home in the brain where they contribute to neurovascular recovery after stroke by promoting restoration of the blood–brain barrier (BBB).^[12]

However, the underlying mechanisms remain to be fully elucidated. Three major mechanistic questions pose as a challenge: first, whether EPCs can release mitochondria; second, whether they can be transferred to brain endothelial cells; and third, whether the transferred mitochondria can restore the brain endothelial cells' functions. Using an *in vitro* stroke model, human EPCs were identified by representative markers including vWF, lectin-UEA, CD34, and Flk-1. EPC-conditioned medium (CM) was collected and after centrifugation, supernatant and particle fractions were analyzed. Western blot analysis showed that the mitochondrial membrane protein TOM40 was enriched in particle fractions, along with higher ATP levels. In addition, Flow cytometry with MitoTracker Red and electron microscopy revealed the presence of mitochondria in the extracellular vesicles released by the EPCs. Interestingly, the oxygen consumption measurement demonstrated that these extracellular mitochondria were functional. Subsequently, the authors examined the media derived from other cell populations, which also revealed vascular function in the neurovascular unit including human astrocytes, endothelial cells, and pericytes. Each cell type's CM was subjected to flow cytometry using three different markers: CD63 as particles marker, MitoTracker Deep Red, and JC-1 to stain mitochondria. Flow cytometry results showed that EPC-derived extracellular particles contained an amount of extracellular mitochondria comparable to those found in the other cellular populations. In addition, EPCs released high levels of extracellular mitochondria with high membrane potentials. Taken together, these findings suggest that human EPCs can be a prolific source of active extracellular mitochondria.

Next step was to answer the second question: Can these mitochondria be transferred? Confocal microscopy demonstrated EPC-derived extracellular mitochondria can be transferred to brain endothelial cells. At this point, the authors tested the effects of EPC-conditioned media on brain endothelial function. A matrigel assay was used to assess the spontaneous formation of capillary-like structures by brain endothelial cells. It showed that both supernatant and particle fractions can promote angiogenesis. Of note, no variation was observed in both empty and ATP-loaded liposome control conditions. In addition to angiogenesis, barrier function equally displays a central function of brain endothelium. Therefore, VE-cadherin and occludin were examined to investigate both adherens and tight junction, respectively. Western blots did not show any difference in protein expression for both molecules. However, immunocytochemistry showed that the particle fraction of EPC media enhanced VE-cadherin membrane localization. Moreover, an endothelial permeability assay was performed by using a transwell system. After the

human brain endothelial cells became confluent on the mesh, fluorescein isothiocyanate-labeled dextran was added to the upper chamber and the fluorescence from the lower compartment was analyzed. EPC-derived particles containing mitochondria decreased brain endothelial permeability, while liposome controls had no effects. Then, the authors asked whether EPC-derived mitochondria may be protective in the brain endothelium cells exposed to an *in vitro* stroke model. Brain endothelial cells were subjected to OGD for 3 h, and then treated with EPC-derived particles. Western blot analysis showed that mitochondrial protein TOM40 was increased in the damaged endothelium and intracellular mitochondrial DNA (mtDNA) and ATP levels were restored after adding EPC particles containing mitochondria along with an improvement in endothelial tightness. To further support these findings, they used a different method to isolate the extracellular mitochondria from EPCs by using fluorescence-activated cell sorting (FACS) of the double-positive MitoTracker Red and PicoGreen EPC-derived extracellular mitochondria. Similar to the previous experimental procedure, the brain endothelial cells were exposed to 3 h of OGD and then treated with FACS-purified mitochondria. Thereafter, also under this condition, treatment with purified extracellular mitochondria reduced endothelial permeability and increased cell viability in the *in vitro* stroke model. In summary, mitochondria released by EPCs can be transferred to the brain endothelium cells, resulting in restoration of mitochondrial functions in an *in vitro* stroke model.

Finally, the final question comes to mind: How does mitochondrial transfer afford protective effects? What is the link between the mitochondrial incorporation and the improvement of endothelium function? The authors tried to answer this intriguing question by performing a proteome analysis of the brain endothelium cells exposed to OGD separating the cells that incorporated the EPCs-derived extracellular mitochondria from the other components. FACS revealed several angiogeneses and BBB-related proteins were upregulated, including bFGF, FGF-4, plasminogen, and Serpin E1 in EPC-derived mitochondria-positive endothelial cells compared to other cells which did not incorporate extracellular mitochondria following OGD. Therefore, a plausible explanation for the observed neuroprotection may be that the mtDNA of transferred mitochondria upregulated specific genes that protected the endothelium. In conclusion, EPCs can release particles containing functional mitochondria and they can be transferred into brain endothelial cells, thereafter promoting angiogenesis and recovery of the BBB function in an *in vitro* stroke model. Altogether, these results suggest that mitochondrial transfer with stem cells such as EPCs can be a potential strategy for

the treatment of stroke and other diseases characterized by mitochondrial dysfunction.

Translational Caveats for Transfer of Healthy Mitochondria after Stroke

The concept of therapeutic mitochondrial transfer from stem cells into the stroke cells represents a novel stroke therapy. A key challenge in this research is fully ascribing neuroprotection to the action of the stem cells' mitochondria that transferred into damaged neurons. To address this caveat, there is a need to control for specificity that neuroprotection is indeed a product of donor mitochondria. In this regard, evidence of specificity should be provided that the healthy mitochondria and the subsequent restoration of cellular bioenergetics within the ischemic tissue are derived from stem cells. Immunofluorescent imaging analyzes *in vitro* and *in vivo* may reveal such distinct transfer of mitochondria into ischemic neurons. Functional assays may involve Clark electrode and Seahorse assays that should reveal the status of mitochondrial function from nontransplanted and transplanted ischemic tissues. Both these outcome parameters are designed to detect microscopically and to functionally assess the healthy mitochondria-mediated restoration of cellular bioenergetics. The transfer of healthy mitochondria from "exogenous" stem cells into "endogenous" ischemic cells may occur acutely posttransplantation but can translate to chronic functional effects, which may explain neuroprotection despite low graft survival in the long term.

In contemplating stem cell-mediated transfer of healthy mitochondria, a critical question that arises is if astrocytes are already transferring mitochondria to ischemic neurons, what further benefit will stem cells provide? We acknowledge the pioneering study by Dr. Lo *et al.*^[9] showing that astrocytes transfer mitochondria to ischemic cells. However, such endogenous transfer is transient and not sufficient to confer robust and stable neuroprotection. Indeed, without exogenous therapeutic intervention, the astrocyte-mediated transfer of healthy mitochondria cannot halt the stroke-induced secondary cell death. Our innovative stem cell-based mitochondria transfer approach will be able to test that transfer of healthy mitochondria through stem cell transplants (EPCs) is a more therapeutically active neuroprotection compared to astrocytes. A *vis-à-vis* analysis of endogenous astrocyte-derived mitochondria (without transplants) and exogenous stem cell-derived mitochondria (with transplants) should reveal the superior efficacy of such stem cell over astrocyte transfer of mitochondria.

In probing mitochondrial function in ischemic cells following transfer of healthy mitochondria from

stem cells, the use of electron transport chain (ETC) Complex I–IV inhibitors should be able to delineate each complex's role in neuroprotection. In addition, an alternative approach to probe that healthy mitochondrial is a central mechanism of neuroprotection is by creating cells with dysfunctional mitochondria, such as Rho0 cells. Evaluating neuroprotection in ischemic cells following coculture or transplantation of EPCs with healthy mitochondria versus Rho0 cells with dysfunctional mitochondria combined with ETC inhibitors should allow critical analyzes of the role of mitochondrial transfer as a neuroprotective mechanism. Of note, we observed that graft survival for both Rho0 and EPCs in stroke brain was modest (<1%) at early time points (1–14 days) and nondetectable at late period (1–3 months), but our thesis is that graft survival *per se* is not a prerequisite, rather transfer of healthy mitochondria by EPCs is key for neuroprotection, which is nullified by Rho0. As for investigating downstream pathways of neuroprotection following transfer of healthy mitochondria, future studies designed to analyze cell death- and cell surviving-associated molecules should further reveal a mechanistic approach in probing healthy mitochondria as mediators of stroke secondary cell death. In this regard, we observed that stroke-altered vasculome genes related to inflammation as highly responsive to transfer of healthy mitochondria.

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Conflicts of interest

There are no conflicts of interest.

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