Exogenous Functional Mitochondria Derived from Bone Mesenchymal Stem Cells That Respond to ROS Can Rescue Neural Cells Following Ischemic Stroke

Lihua Dai1,*, Zheqian Wu1,*, Liili Yin1, Longjian Cheng1, Qiang Zhou2, Fei Ding1

1Department of Emergency, Shidong Hospital, ShangHai, People’s Republic of China; 2Department of General Surgery, Eighth People’s Hospital, ShangHai, People’s Republic of China

*These authors contributed equally to this work

Correspondence: Fei Ding; Qiang Zhou, Email 10119038@qq.com; rabbit1264@163.com

Background: Upon uptake by stressed cells, functional mitochondria can perform their normal functions, ultimately enhancing the survival of host cells. However, despite the promising results of this approach, there is still a lack of understanding of the specific relationship between nerve cells and functional mitochondria.

Methods: Functional mitochondria (F-Mito) were isolated from bone marrow-derived mesenchymal stem cells (BMSCs). The ability of microglia cells to internalize F-Mito was evaluated using a middle cerebral artery occlusion (MCAO) model in C57BL/6J mice and an oxygen-glucose deprivation/reoxygenation (OGD/R) cell model. After OGD/R and F-Mito treatment, the temporal dynamics of intracellular reactive oxygen species (ROS) levels were examined. The relationship between ROS levels and F-Mito uptake was assessed at the individual cell level using MitoSOX, Mitotracker, and HIF-1α labeling.

Results: Our findings indicate that microglia cells exhibit enhanced mitochondrial uptake compared to astrocytes. Furthermore, internalized F-Mito reduced ROS levels and HIF-1α levels. Importantly, we found that the ROS response in microglia cells following ischemia is a critical regulator of F-Mito internalization, and promoting autophagy in microglia cells might reduce the uptake of ROS and HIF-1α levels.

Conclusion: It is verified that F-Mito derived from BMSCs play a protective role in ischemia-reperfusion injury, as their weakening reduces microglial cell activation and alleviates neuroinflammation.

Keywords: neuroinflammation, ROS, stem cell, HIF-1α, ischemic stroke

Introduction

Stroke is one of the leading causes of death and disability globally, second only to ischemic heart disease. Stroke has become a major cause of premature death and disease burden. The treatment for acute ischemic stroke aims to restore perfusion to brain tissue and can be achieved through thrombolytic drugs or endovascular therapy. However, only a small percentage of stroke patients actually receive acute treatment. Administering thrombolytic therapy within 4.5 hours of stroke onset is the most effective treatment for acute ischemic stroke.1,2 Research suggests that approximately 25% of ischemic stroke patients are eligible for thrombolytic therapy, and 10–12% are suitable for endovascular treatment. Without treatment intervention, the ischemic area may expand over time to surrounding areas, leading to additional cell death in the days or weeks following stroke and infarction. Although treatment within the effective window can reduce the mortality rate after ischemic stroke, over 60% of stroke survivors still face varying degrees of motor, cognitive, language, and swallowing impairments. These consequences can severely impair their ability to care for themselves, lower their quality of life, and increase the burden on their families and society. Therefore, there is an urgent need for...
The administration of bone marrow stem cells emerges as a promising novel approach in addressing nerve damage resulting from hypertension and stroke. Prior research has demonstrated the potential of bone marrow stem cells to enhance the rehabilitation of limb dysfunction in individuals affected by ischemic stroke, while also mitigating concurrent dysfunctions.

Mitochondria demonstrate significant functional adaptability within cells to meet local metabolic demands. Observations have shown the internalization of exogenous mitochondria in microglia and endothelial cells following an ischemic stroke, promoting the survival and activity of endogenous cells. Nevertheless, the precise uptake of exogenous mitochondria by microglia cells after an ischemic stroke remains unclear.

Recent research has revealed that cellular oxidative stress is a prerequisite for the transfer of mitochondria within the cell. Under conditions of glucose and oxygen deprivation, followed by reoxygenation, endothelial or myocardial cells can acquire mitochondria from MSCs to improve their survival. Similarly, paclitaxel treatment increases the transfer of mitochondria from MSCs to injured endothelial cells, while ethidium bromide treatment enhances the transport of mitochondria from mesenchymal stem cells to treated osteosarcoma cell. However, the mechanisms underlying the acquisition of exogenous functional mitochondria by damaged cells and the molecular signals that regulate this process remain unclear. To fill this research gap, this study used an ischemic stroke model to evaluate the therapeutic effects of exogenous functional mitochondria and explore its potential mechanism of action.

**Materials and methods**

**Isolation and Culture of Bone Marrow Mesenchymal Stem (BMSCs)**

Bone marrow stromal cells (BMSCs) were isolated from 2-month-old ICR and 6-month-old C57/B6L mice following the detailed procedures outlined in a previous study. The bone marrow was aseptically extracted by flushing the femurs and tibias of the mice and then suspended in low-glucose DMEM (Hyclone) supplemented with 10% FBS (Gibco) in a humidified incubator (Thermo) containing 95% air and 5% CO₂ at 37°C. Non-adherent cells were removed, and the medium was changed after 3 days, with subsequent changes every 2 days. The cells were passaged for use in further experiments.

**Primary Astrocytes and Microglia Cultures**

Primary cultures of astrocytes and microglia were obtained from the cortex of neonatal C57BL/6J mice at 2 days of age. The cortical cells were suspended in DMEM supplemented with 25mM glucose, 4mM glutamine, 1mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. Subsequently, the cells were seeded at a density of 6×105 cells/cm² in uncoated 25cm² flasks. After approximately 14 days, a monolayer of astrocytes had formed. The non-astrocytic cells, including microglia and neurons, were separated through shaking and medium replacement. Upon reaching a density of 70%-80%, the astrocytes were dissociated using trypsin and replated in uncoated T75 flasks.

**Mitochondrial Labeling in Cells and Isolation of Functional Mitochondria from Bone Marrow Mesenchymal Stem Cells (BMSCs)**

BMSCs were labeled with HBLV-mito-dsred-Null-PURO (LV60102279, Hanbo) or Mito-tracker red (Beyotime) for mitochondrial visualization. Functional mitochondria (F-Mito) were isolated from BMSCs using the Mitochondria Isolation Kit for cultured cells (Solarbio, #SM0020-50T) and characterized based on the provided protocols. The isolated F-Mito was immediately stored on ice and used under light avoidance conditions.

**In vitro Oxygen-Glucose Deprivation and Reoxygenation (OGD/R) and F-Mito Treatment OGD/R Experiments**

In vitro experiments involving oxygen-glucose deprivation and reoxygenation (OGD/R) as well as the treatment with F-Mito during OGD/R were carried out in a 37°C constant temperature incubator under an anaerobic atmosphere of 95% N₂ and 5% CO₂. Before subjecting them to OGD/R, the cells were labeled with Hoechst 33,342 or 1μg/mL of WGA (W7024, Invitrogen). OGD/R was induced by exposing the cells to an OGD solution lacking oxygen and glucose.
2.5 hours of incubation, the culture medium was replaced with normal medium containing high-glucose DMEM and 10% FBS (HD), or with medium containing uniformly distributed F-Mito, or with medium containing 1.2mM of N-acetylcysteine (NAC, MedChemExpress). F-Mito was isolated from 3.4 to 3.8×10⁵ BMSCs, which correspond to 2.5x10⁴/cm² of astrocytes, and microglia cells. Subsequent to OGD/R, the cells were allowed to recover for 3 hours in a standard incubator before further experiments were conducted.¹⁴

**Transmission Electron Microscopy (TEM) Method**

The samples were fixed in 2.5% glutaraldehyde in 0.1M PB (PH7.2) overnight, then washed three times for 7 minutes in 0.1M PB (PH7.2). Subsequently, the samples were fixed for 2 hours with 1% OsO₄, washed three times for 7 minutes in ddH₂O, and then dehydrated and embedded using Epon 812 resin. Thin sections were prepared using a Leica EM UC7 ultramicrotome, stained with uranyl acetate and lead citrate, and observed using a JEM-1400 Plus transmission electron microscope at 80kv.¹⁵

**Mitochondrial Membrane Potential Measurement**

The JC-1 dye (Invitrogen) was employed to evaluate the mitochondrial membrane potential as an indicator of mitochondrial health. BMSCs and F-Mito were incubated with JC1 (5μM or 1μM) for 20 minutes at 37°C. JC1 accumulates within the mitochondria in a manner dependent on the potential, leading to a change in fluorescence emission from green (Ex 485nm/Em 516nm) to red (Ex 579nm/Em 599nm). The mitochondrial membrane potential (MMP) was subsequently assessed using a SLIDEVIEW microscope for both BMSCs and F-Mito.¹⁶

**Detection of ROS Generation**

Cell-permeable 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was utilized to quantify intracellular levels of ROS through fluorescence intensity measurement, with excitation and emission wavelengths set at 488nm and 525 nm, respectively, using a microplate reader (Infinite M200 pro, TECAN). ROS detection in brain tissue was performed using confocal laser scanning microscopy (CLSM). MitoSOX™ Red staining was employed to evaluate mitochondrial ROS production in microglia cells, and astrocytes, cultured on pre-coated cell climbing slides (Solarbio, YA0350). After treatment, the cells were washed with PBS and incubated at 37°C for 30 minutes. Subsequently, the cells were washed three times with PBS at room temperature before examination using SLIDEVIEW.¹⁷

**Focal Ischemic Stroke**

Transient middle cerebral artery occlusion (MCAO) was induced in male mice (8–10 weeks old) following established procedures for randomization and blinding as described by Wang.¹⁸ The researchers inserted an intraluminal filament into the right internal carotid artery to induce localized cerebral ischemia. After 30 minutes of ischemia, the filament was removed, and the mice were allowed to recover. Subsequently, the animals underwent behavioral testing, magnetic resonance imaging, and immunofluorescent staining at specific time points.

**Mitochondrial Treatment in an in vivo Stroke Model**

Functional mitochondria were isolated from 5×10⁶ BMSCs per mouse and administered into the hippocampus using a stereotaxic apparatus (RWD, 71,000-M, China). The injection comprised a 4μL suspension of F-Mito or saline solution.¹⁹

**Immunofluorescent Staining**

The mice brains were extracted and fixed in 4°C PFA overnight, then gradually transferred to higher sucrose concentrations for 48–72 hours. Subsequently, the brains were sectioned into 20μm coronal slices and subjected to immunofluorescent staining using primary antibodies against GFAP, Iba1, and HIF-1α (Abcam ab7260, ab15690 and ab179483), followed by incubation with secondary antibodies and DAPI staining. The stained sections were then imaged using an LSM 800 confocal microscope.²⁰
Mirrors Water Maze
Equipment preparation: Prepare a large circular water tank with a diameter usually ranging from 1.2 to 1.5 meters and a depth of approximately 0.6 meters. The tank should have a small platform with a diameter of about 10 to 15 centimeters. The tank needs to be filled with water, maintaining a temperature of 20–22 degrees Celsius. To make the platform less detectable, non-toxic dye can be added to the water. Training phase: Place the experimental animal in a random location within the water tank and allow it to freely swim and search for the platform. Once the animal finds the platform, it should stay on it for 10–20 seconds to establish memory. If the animal cannot find the platform within a predetermined time (eg, 60 or 120 seconds), the experimenter should guide it to the platform. This training should be conducted 4–5 times a day, with sufficient rest periods between each session.21

Statistical Analysis
Three independent experiments (n = 3) were conducted, and statistical analysis was carried out using SPSS 20.0. Pairwise comparisons were evaluated using Student’s t-test, while multiple comparisons were assessed using one-way analysis of variance (ANOVA) with Bonferroni correction. A p-value < 0.05 was considered statistically significant.22

Results
F-Mito Improved Neurological Outcomes After MCAO
In the MCAO group, compared to the control group, latency for seeking, the percentage of time spent in target zone III, and total distance from the platform increased noticeably at 1, 2, 3, and 4 days (Figures 1A–C, *P<0.05), and the number of times rats crossed the original platform significantly reduced at 5 days (Figure 1D–G). Nevertheless, F-Mito ameliorated the changes in latency for seeking and total distance from the platform (#P<0.05).

The Cellular Uptake of F-Mito in the Brain Following an Ischemic Stroke Was Investigated
We examined the internal uptake of fluorescently labeled mitochondria (F-Mito) in various cell types relevant to the restoration of neurological function following an ischemic stroke. Analysis using confocal microscopy demonstrated the localization of reactive oxygen species (ROS) in microglia cells (Figure 2A–J) and astrocytes (Figure 2K–T) near the injection site. Three days after middle cerebral artery occlusion (MCAO), the group treated with F-Mito exhibited a marked reduction in ROS.

Expression of SDH in the Cerebral Cortex at 3 Day After MCAO
We used succinate dehydrogenase (SDH) and an electron microscope to assess mitochondrial damage and detect axonal injury in the cerebral cortex. SDH expression levels were detected in sporadic cells and were confirmed to be in astrocytes using double labeling with Iba-1 staining. The increase in SDH immunoreactivity in microglial cells after MCAO (Figures 3D–F) compared to the control samples (see Figures 3A–C) was reversed by treatment with F-Mito (Figures 3G–3I). The trend of SDH expression in GFAP-labeled astrocytes is similar to that F-Mito can reduce SDH expression (Figures 3K–T). Electron microscopy showed an increase in mitochondrial damage at day 3 compared to the control group (Figures 3U and V), and F-Mito mitigated the mitochondrial damage (Figure 3W).

LC3B Protein Expression Levels in the Cerebral Cortex
Post MCAO, a reduction in the levels of LC3 was evident in rats, as depicted in Figure 4B. Notably, pretreatment with F-Mito led to the restoration of LC3 levels in the cortex 3 day after MCAO, as indicated in Figures 4A–I. Analysis via transmission electron microscopy showed that, at 3 day post MCAO, astrocytes exhibited reduced cytoplasmic vacuolization compared to the control group. These vacuoles contained either amorphous or electron-dense material and were encased by a single or double membrane, F-Mito demonstrated the capability to reverse this phenomenon, as indicated in Figure 4J–4L.
The F-Mito Restored Cell Viability and Reduced Intracellular ROS Following OGD/R in Astrocytes

Two distinct types of fluorescent dyes were employed to quantify the levels of reactive oxygen species (ROS) in this investigation. Astrocytes exposed to OGD/R exhibited a noteworthy escalation in intracellular ROS levels at 3 hours, as opposed to the control group cultured under normal conditions, as depicted in Figure 5A–C. Additionally, MitoSOX staining depicted heightened ROS levels at 3 hours after the OGD/R challenge, as demonstrated in Figure 5D–F. The administration of F-Mito led to a reduction in ROS levels at 3 hours relative to untreated cells (Figure 5G–J and K–T).

The F-Mito Restored Cell Viability and Reduced JC-1 Following OGD/R

Two different fluorescent dyes were utilized for JC-1 level measurement. Specifically, astrocytes and microglia cells exposed to OGD/R exhibited a notable rise in intracellular JC-1 levels (Figure 6D–F) at 3 hours in comparison to the control group cultured under normal conditions (Figure 6A–C). Conversely, treatment with F-Mito led to a reduction in JC-1 levels at 3 hours relative to untreated cells (Figure 6G–J and K–T).

Figure 1 F-Mito Improved Neurological Outcomes after MCAO. In the MCAO group, compared to the control group, the latency for seeking, the percentage of time spent in target zone III, and the total distance from the platform notably increased at 1, 2, 3, and 4 days (A–C), while the number of times rats crossed the original platform significantly reduced at 5 days (D–G). Additionally, F-Mito mitigated the changes in latency for seeking and total distance from the platform. N=3. *P < 0.05.
The expression level of LC3B was lower 3 h after OGD compared to the control group. F-Mito significantly improved the expression level of LC3B (Figures 7A–J). The expression trend of HIF-1α was opposite to that of LC3B (Figures 7K–T). There was a significant increase in HIF-1α optical density 3 h after OGD/R compared to the control group. However, F-Mito can reverse this effect.

**F-Mito Regulates Oxidative Stress by Promoting Autophagy in vitro**

The expression level of LC3B was lower 3 h after OGD compared to the control group. F-Mito significantly improved the expression level of LC3B (Figures 7A–J). The expression trend of HIF-1α was opposite to that of LC3B (Figures 7K–T). There was a significant increase in HIF-1α optical density 3 h after OGD/R compared to the control group. However, F-Mito can reverse this effect.

**Discussion**

Lacunar infarcts are a common subtype of ischemic strokes and could be the initial indication of cerebral small vessel disease, a progressive condition affecting the small arteries, capillaries, and venules in the brain. The pathophysiological
Figure 3 Expression of SDH in the cerebral cortex at 3 day after MCAO. The expression levels of SDH were observed in isolated cells and were subsequently confirmed to be present in astrocytes through double labeling with Iba-1 staining. Following MCAO, an increase in SDH immunoreactivity was observed in microglial cells (D–F), compared to control samples (A–C). Treatment with F-Mito reversed the observed increase in SDH immunoreactivity (G–J). The pattern of SDH expression in GFAP-labeled astrocytes mirrored that F-Mito demonstrated the ability to reduce SDH expression (K–T). Furthermore, electron microscopy indicated a rise in mitochondrial damage on day 3 in comparison to the control group (U and V), with F-Mito ameliorating the mitochondrial damage (W). N=3. *P < 0.05, **P < 0.01, ***P < 0.005.
processes behind artery occlusion in these cases are complex and not yet fully understood, partly due to limitations in neuroimaging resolution, scarcity of pathological research, and absence of reliable experimental models. There is evidence to suggest that stroke or transient ischemic attack is the primary manifestation of hematological diseases. Among them, 12 patients had ischemic strokes, which is an interesting finding. Inflammation presents a viable target for stroke secondary prevention, especially relevant to small vessel disease (SVD) etiology as other mechanisms besides thrombosis play a crucial role beyond the acute stroke phase. The URICO-ICTUS trial demonstrated encouraging outcomes regarding the efficacy of uric acid, a potent endogenous antioxidant, in conjunction with systemic thrombolysis for patients with acute ischemic stroke.

Mitochondria, often referred to as the “energy factory”, are vital organelles within the cytoplasm, crucial for ATP generation and maintaining cellular homeostasis. However, the mechanisms underlying the initiation and facilitation of exogenous mitochondrial transfer remain a subject requiring further investigation. Reactive oxygen species (ROS) are produced as by-products during the ongoing redox process within the mitochondria. Environmental stress can lead to a significant increase in ROS levels, causing oxidative stress and damage to cellular proteins, lipids, and DNA. Studies have shown that ROS plays a crucial role in removing dysfunctional endogenous mitochondria through migrasome or...
mitophagy processes. Additionally, membrane internalization-related proteins like clathrin and integrin-mediated src signaling are vital for mitochondrial internalization. In conclusion, this study provides evidence that ROS levels act as an early response in facilitating the internalization of functional mitochondria to restore cell viability after ischemic stroke, offering insights into the precise regulation of mitochondrial transplantation and the development of effective therapeutic techniques for ischemic stroke.

When cerebral infarction occurs, oxidative stress refers to a series of biological effects caused by the imbalance of cellular redox state when the internal and external environments of the cells undergo changes. Specifically, when the
brain tissue is ischemic and hypoxic, a large amount of reactive oxygen species are produced, which will damage cell membranes, mitochondria, and other cell structures, ultimately leading to cell death. At the same time, ischemia and hypoxia in brain tissue can also affect the redox state inside the cells, leading to the occurrence of oxidative stress. In addition, the inflammatory response after cerebral infarction can exacerbate oxidative stress.\textsuperscript{38-30} The inflammatory response will lead to the infiltration and chemotaxis of a large number of inflammatory cells, which will release a large amount of reactive oxygen species and inflammatory factors, further aggravating cell damage and death. We found that the increase in SDH and ROS immunoreactivity in microglial cells after MCAO compared to the control samples was reversed by treatment with F-Mito.

Autophagy is a lysosome-dependent process that preserves cellular homeostasis by degrading and recycling organelles and proteins for energy. Inhibiting autophagy may lead to neuroinflammation.\textsuperscript{31} The findings showed a notable decrease in LC3 levels in the cerebral cortex 3 days after MCAO. F-Mito increased the expression of LC3 after 3 days,
whereas a contrary trend was observed for p62 levels. An electron microscope is best utilized in combination with other methods to ensure a comprehensive approach, which is increasingly crucial for advancing autophagy research. The data suggested a reduction in the number of autophagic bodies in astrocytes 1 day after ischemia-hypoxia. These results suggested that F-Mito could mitigate mitochondrial damage and neuroinflammation through modulation of autophagy, although the specific molecular target remains unidentified. In vitro findings showed upregulation of HIF-1α, mitoSOX, and SDH expression levels 1 hour after OGD. Autophagy regulates astrocyte activation, playing a crucial role in sustaining astrocytic functions.

JC-1 is a commonly used membrane potential fluorescent probe for detecting changes in cell membrane potential. It works by injecting fluorescent dyes into the cells, and then observing the fluorescent signals emitted by the dyes using equipment such as a fluorescence microscope or flow cytometer, in order to determine the state of the cell membrane potential. In pathological conditions such as brain ischemia and hypoxia, the cell membrane potential changes, resulting
in changes in the fluorescent signals emitted by the JC-1 dye.\textsuperscript{27,32} Therefore, JC-1 dye can be used to monitor the changes in cell membrane potential during the pathological processes of brain ischemia and hypoxia, providing strong support for further research on the mechanisms and treatment of brain ischemia. Our research found that astrocytes and microglia cells exposed to OGD/R showed a significant increase in intracellular JC-1 levels at 3 hours compared to the control group cultured under normal conditions. In contrast, treatment with F-Mito resulted in a decrease in JC-1 levels at 3 hours compared to untreated cells.

These results reveal the mechanism by which exogenous mitochondria rescue nerve cells through ROS response in an ischemic stroke model. Overall, our study provides reliable evidence for the promising therapeutic potential of mitochondria from BMSCs as a treatment for ischemic stroke.

**Data Sharing Statement**
Publicly available datasets were analyzed in this study.

**Ethics Approval and Consent to Participate**
Approval of the Laboratory Animal Ethical and Welfare Committee (Shidong Hospital Ethics Committee): SDEC-2023-002-01. Complying with the five freedoms (freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behavior, freedom from fear and distress) and the 3Rs principle (replacement, reduction, refinement for experimental animals) (hence death is an unacceptable endpoint for experimental animals).

**Author Contributions**
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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