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Primary astrocytic mitochondrial transplantation ameliorates ischemic stroke

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Mitochondria are important organelles that regulate adenosine triphosphate production, intracellular calcium buffering, cell survival, and apoptosis. They play therapeutic roles in injured cells via transcellular transfer through extracellular vesicles, gap junctions, and tunneling nanotubes. Astrocytes can secrete numerous factors known to promote neuronal survival, synaptic formation, and plasticity. Recent studies have demonstrated that astrocytes can transfer mitochondria to damaged neurons to enhance their viability and recovery. In this study, we observed that treatment with mitochondria isolated from rat primary astrocytes enhanced cell viability and ameliorated hydrogen peroxide-damaged neurons. Interestingly, isolated astrocytic mitochondria increased the number of cells under damaged neuronal conditions, but not under normal conditions, although the mitochondrial transfer efficiency did not differ between the two conditions. This effect was also observed after transplanting astrocytic mitochondria in a rat middle cerebral artery occlusion model. These findings suggest that mitochondria transfer therapy can be used to treat acute ischemic stroke and other diseases. [BMB Reports 2023; 56(2): 90-95]

INTRODUCTION

Ischemic stroke occurs due to a blockage in the cerebral artery by thrombosis or embolism. Stroke is one of the leading causes of serious disability in adults. Approximately 80-90% of strokes are ischemic. Oxidative stress is a crucial factor in ischemia-reperfusion injury (1, 2).

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Mitochondria are double-membraned cytoplasmic organelles that generate the majority of adenosine triphosphate (ATP) *via* oxidative phosphorylation to control vital physiological processes, including metabolism and cellular homeostasis. In addition, mitochondria are responsible for intracellular calcium buffering, apoptosis, and production of reactive oxygen species (ROS), which are essential for cell development, function, and survival (3-5).

In recent years, many studies have shown that mitochondrial oxidative damage can greatly influence various neurodegenerative diseases. Mitochondrial transfer has been proposed as a therapeutic strategy in such cases (6, 7). Under stressful conditions, damaged mitochondria are substituted by new ones through transcellular transfer *via* tunneling nanotubes (TNTs), extracellular vesicles (EVs), and gap junctions (GJs). Mitochondria can be obtained from any organ and cell type of the body. For example, transfer of isolated mitochondria from mesenchymal stem cells (MSCs) can effectively treat diabetic nephropathy (DN) and acute ischemic stroke (8-10).

Astrocytes are the most abundant cell type in the central nervous system (CNS) (11). They can secrete factors to promote synapse formation, metabolic activity, neurotrophy, and plasticity (12, 13). Astrocytes can release extracellular mitochondrial particles and transfer mitochondria into neurons after stroke in a CD38-dependent manner (14).

To further develop astrocytic mitochondrial transfer as a novel therapeutic option for stroke, we demonstrated that exogenous astrocytic mitochondrial transfer could affect oxidative stress-induced neuronal damage in middle cerebral artery occlusion (MCAO) animal models. Additionally, we found that astrocytic mitochondrial treatment had potential applications in other neurological disorders such as Parkinson's disease (PD).

RESULTS

Neuronal recovery after isolated astrocytic mitochondrial transfer

In the brain, astrocytes support neuronal function by secreting astrocyte-derived extracellular vesicles (13). Because astrocytic vesicles have various contents including mitochondria, we investigated effects of astrocytic mitochondria on neural lineage cells. First, we added a red fluorescent probe, MitoTracker Red

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CMXRos, to postnatal day 5 (P5) rat cortical astrocytes to stain the mitochondria (Fig. 1A, B). We then isolated mitochondria from astrocytes using sucrose-HEPES-EGTA (SHE) buffer to treat embryonic day 14 (E14) rat cortical neural progenitor cells (NPCs). We observed that a large proportion of mitochondria entered recipient cells (Fig. 1C, D), indicating that isolated astrocytic mitochondria could be successfully transferred into neural lineage cells.

Astrocytes can release extracellular mitochondrial particles via CD38-mediated mechanisms. These particles can enter damaged neurons to protect and restore them (14). Therefore, we analyzed effects of isolated astrocytic mitochondria on hydrogen peroxide (H_2O_2)-damaged neurons (Fig. 2A). H_2O_2 is commonly used in *in vitro* stroke models (15, 16). Isolated mitochondria (red) were co-expressed with class III beta-tubulin (TUJ1)-positive cells to induce efficient mitochondrial transfer in differentiated neurons (Fig. 2B). We treated differentiating neurons on the 7th day (both normal and H_2O_2-damaged) twice with mitochondria and evaluated TUJ1-positive cells on day 17. The mitochondrial treatment group showed significant recovery following H_2O_2 neurotoxin application (Fig. 2C-E). Remarkably, when mitochondria were transferred under normal conditions, they had no neurotrophic effect on neurons.

We isolated mitochondria by crushing them using syringe approximately 90-100 times (30 times/30 s off/30 times/30 s off/30-40 times). To obtain fresh mitochondria quickly, we used a sonicator instead of a syringe (5 s/5 s off/5 s). Both methods of mitochondrial isolation led to successful delivery into target cells (Fig. 2F).

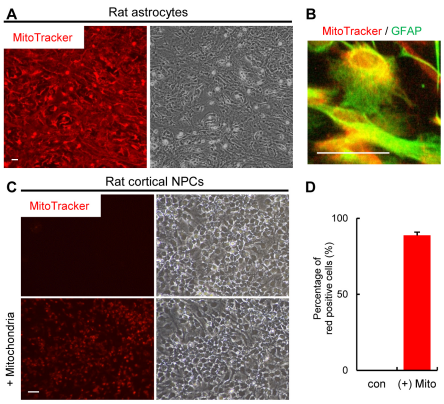

Fig. 1. Isolated astrocytic mitochondrial transfer into NPCs. (A, B) Using MitoTracker Red dye, mitochondria in rat P5 astrocytes (A) and GFAP-positive (astrocytic marker) cells (B) were stained. (C, D) Isolated mitochondria from astrocyte-treated rat cortical NPCs. Immunofluorescence shows MitoTracker-labeled astrocytic mitochondria (red) in NPCs (C). The graph shows mitochondrial transfer efficiency (D). Error bars, standard error (S.E.); Scale bar, 20 μm. GFAP, glial fibrillary acidic protein; NPC, neural progenitor cell.

Astrocytic mitochondrial transfer enhances therapeutic effects in an *in vivo* ischemic stroke model

To examine whether transfer of isolated healthy astrocytic mitochondria had therapeutic effects in *in vivo* ischemic stroke disease models, we stereotaxically injected mitochondria into the striatum of MCAO rats. Mitochondrial transplantation was performed twice 24 h apart. Animals in the control group were injected with saline (Fig. 3A). Twenty-four hours after transplantation, we sliced the brain and confirmed the presence of transplanted astrocytic mitochondria, indicating a successful transfer (Fig. 3B, C). Moreover, the infarct volume of mitochondria injected into MCAO rat model was significantly lower than that in the saline-injected model (Fig. 3D, E). These results demonstrate that astrocytic mitochondrial transfer has therapeutic effects on ischemic stroke.

Isolated astrocytic mitochondria also improve other neurological disorders

PD is a prevalent neurodegenerative disorder caused by selective loss of dopamine (DA) neurons in the substantia nigra (17, 18). We tested whether astrocytic mitochondria could aid recovery from other neurological disorders such as PD. We used

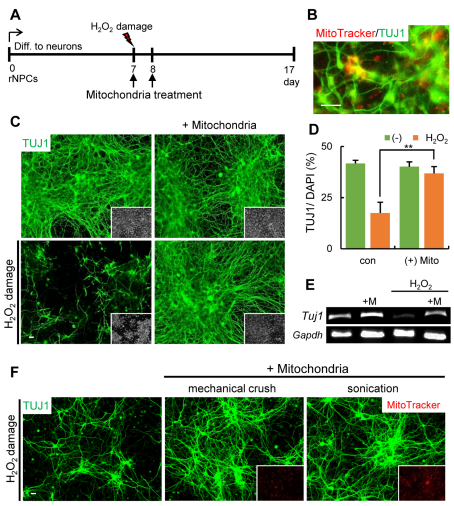

Fig. 2. Mitochondrial transfer enhances recovery after neural damage. (A) Schematic illustration of experimental timeline. (B) MitoTracker-labeled astrocytic mitochondria transferred into neurons (TUJ1-positive cells). (C-E) After differentiation day 7 neurons were induced with 100 μM H_2O_2 for 3 h to show neurotoxic damage, cells were treated with isolated astrocytic mitochondria twice. Immunofluorescence staining (C) and quantification of TUJ1-positive neurons (D) are shown. (E) RT-PCR analysis of *Tuj1* mRNA in mitochondria-treated cells. *Gapdh* expression was used as a loading control. (F) Comparison of different methods of mitochondrial isolation. Mechanical crushing was performed 30 times/30 s off/30 times/30 s off/30 times and sonication was performed 5 s/5 s off/5 s. Error bars, standard error (S.E.). **P < 0.01. Scale bar, 20 μm. TUJ1, class III beta-tubulin; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

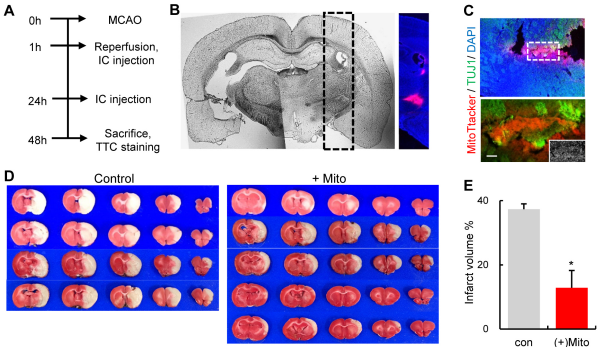

Fig. 3. Effect of mitochondrial transplantation in an MCAO model. (A) Schematic illustration of experimental timeline. (B) Astrocyte-derived MitoTracker-labeled mitochondria (red) in the injected area (48 h). (C) Immunofluorescence images of TUJ1 expression and localization of injected MitoTracker-labeled mitochondria (red). White square highlights colocalization of MitoTracker and TUJ1 expression. (D) Typical TTC staining results. (E) Quantitation of infarct volumes. Error bars, standard error (S.E.). *P $<$ 0.05. Scale bar, 200 μm. TUJ1, class III beta-tubulin; TTC, 2,3,5-triphenyltetrazolium chloride.

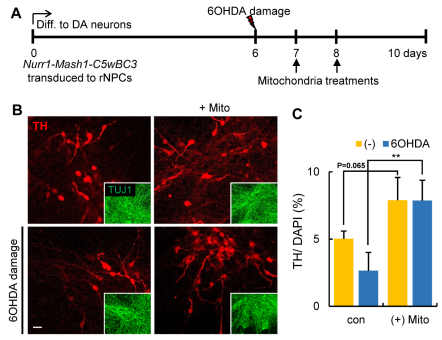

Fig. 4. Astrocytic mitochondria enhance survival of DA neurons following 6-OHDA toxicity. (A) Schematic illustration of experimental timeline. (B, C) After performing neurotoxic insult with 20 μM 6-OHDA for 18 h to *Nurr1-Mash1*-overexpressing rat DA neurons, cells were treated with isolated astrocytic mitochondria twice. Immunofluorescence staining (B) and quantification of TH-positive neurons (C) are shown. Error bars, standard error (S.E.). **P $<$ 0.01. Scale bar, 20 μm. DA, dopamine; 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase.

Nurr1-Mash1 retrovirus to induce DA neurogenesis at differentiation day 6 and exposed them to 6-hydroxydopamine (6-OHDA, 20 μM, 18 h) to induce DA neuronal damage (19, 20). Isolated astrocytic mitochondrial treatments were performed twice. DA neurons were fixed at 2 days after transfer (Fig. 4A). We observed that treatment with astrocytic mitochondria rescued DA neurons compared with the control (Fig. 4B, C). These results suggest that therapeutic effects of astrocytic mitochondria might be applied to various neurological disorders apart from ischemic stroke.

DISCUSSION

Transfer of functional exogenous mitochondria into damaged cells can replace existing mitochondria with new ones. Transplantation of isolated mitochondria is a novel therapeutic strategy for treating mitochondrial dysfunction (7, 21). Ischemic stroke occurs when blood vessels supplying blood to the brain are obstructed (1). Mice with ischemic stroke show mitochondrial fission, morphological changes in the mitochondria, high levels of free radicals, and ATP depletion (22, 23). A recent study has suggested that astrocytic mitochondria can enter damaged cortical neurons and recover neuronal dendrites and ATP levels after an ischemic stroke (14, 24).

In this study, to assess therapeutic application of mitochondrial transfer, we isolated mitochondria from primary rat astrocytes and used them to treat damaged neurons. These isolated mitochondria were successfully transferred to target cells (Fig. 1). Exogenous mitochondria remained in cells for a long time, positively affecting them. In addition, the isolated mitochondria effectively induced an increase in cell number during H_2O_2 neurotoxin damage (Fig. 2). Interestingly, these effects were observed only under damaged condition, showing no effect on neurons under normal conditions. This may be because existing mitochondria from damaged neurons were replaced by exogenous mitochondria after mitochondrial transfer. Next, we examined whether astrocytic mitochondria relieved stroke symptoms *in vivo*. Freshly isolated mitochondria from cultured astrocytes were injected into striatum of MCAO rats (100 μg/rat). Before mitochondrial isolation, astrocytic mitochondria were labeled with MitoTracker Red to determine the graft success rate. We observed that mitochondria-injected rats had smaller infarct sizes than saline-injected rats (Fig. 3). A greater proportion of saline-injected rats died than that of mitochondria-injected rats (data not shown). This suggests that transfer of astrocytic mitochondria could provide therapeutic benefits to ischemic stroke patients. Moreover, we investigated the effect of mitochondria on PD *in vitro* using DA neurons damaged by 6-OHDA (a specific DA neurological toxin). Previous studies have demonstrated that impaired mitochondrial function can lead to oxidative stress in dopaminergic cells and that mitochondrial dysfunction plays a central role in the pathogenesis of PD (25). We added 6-OHDA (20 μM) for 18 h to DA neurons on differentiation day 6. Astrocytic mitochondria were used for treatment twice after neurological damage in the same manner as in previous experiments. Results showed that astrocytic mitochondrial transfer had a recovery effect on PD (Fig. 4). However, the recovery effect on 6-OHDA-damaged DA neurons was not as significant as that on H_2O_2-damaged cortical neurons. We speculate that the exchange of healthy mitochondria has a greater influence on ischemic stroke than on PD.

In conclusion, we demonstrate that transfer of mitochondria isolated from healthy primary astrocytes has a positive effect on the survival and recovery of damaged neurons. In addition,

in a rat ischemic stroke model, astrocytic mitochondrial transplantation reduced the infarct size compared with that in control rats. Mitochondrial transplantation therapy still has limitations in terms of quality control and long-term storage of isolated mitochondria. Further investigation is required to improve them. Overall, our results suggest that transferring astrocyte-derived mitochondria might represent a novel therapeutic approach for treating stroke and other neurological disorders.

MATERIALS AND METHODS

Primary rat astrocyte culture

The animals were housed and treated according to the Institutional Animal Care and Use Committee (IACUC, 2021-0065A) guidelines of Hanyang University, Korea. After removing the meninges, cerebral cortical tissue from Sprague Dawley (SD) rats (DaeHan BioLink, Seoul, Korea) was dissected and dissociated mechanically on P5. Cells were plated in 75-cm^2 T-flasks, and the growth medium was composed of DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 8% horse serum (HS; Thermo Fisher Scientific), 50 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), penicillin/streptomycin (Thermo Fisher Scientific), B27 (Thermo Fisher Scientific), Glutamax (Thermo Fisher Scientific), and 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA). When cell confluency reached 90%, minor microglia were removed by gentle shaking, and pure astrocytes were harvested and replated in poly-L-ornithine (PLO; 15 μg/ml, Sigma-Aldrich)/fibronectin (FN; 1 μg/ml, Sigma-Aldrich)-coated dishes.

Primary rat NPC culture

After removing the embryos from female SD rats on E14, the cerebral cortex was dissected, and single cells were isolated from the tissue. NPCs were seeded onto plates coated with PLO/FN. The growth medium was composed of a serum-free medium (N-2), to which 20 ng/ml bFGF was added. Confluent cells were incubated for 1 h in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) for single-cell dissociation. After dissociation, cells were plated on PLO/FN-coated glass slides in 24- or 6-well plates. For neuronal differentiation, bFGF was withdrawn from the medium, and 0.2 mM ascorbic acid (Sigma-Aldrich) was added.

Isolation of mitochondria

To label the mitochondria, rat astrocytes were incubated with 0.5 μM MitoTracker Red CMXRos (Invitrogen) for 20 min. After staining, we washed the cells with phosphate-buffered saline (PBS), trypsinized them for 5 min, and harvested the cell pellet. The cell pellet was then resuspended in 0.2 ml SHE buffer (0.25 M sucrose, 20 mM HEPES (pH 7.4), 2 mM EGTA). After incubation on ice for 5 min, the pellet was homogenized using a 1 ml syringe (26-gauge needle) 100 times (30/30/40) or sonicated for 10 s (5/5) and centrifuged at 1,100 × g for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 12,000 × g for 15 min at 4°C. After removing the supernatant, the mitochondrial pellet was washed with 1 ml SHE buffer, centrifuged at 20,000 × g for 5 min at 4°C, and resuspended in 50 μl of 20 mM HEPES (pH 7.4). The concentration of isolated mitochondria was quantified using a Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific).

Neurotoxin treatments

To determine the recovery effects of mitochondrial treatment, H_2O_2 (100 μM, 3 h; Sigma-Aldrich) was added to the differentiation day 7 neurons. 6-OHDA (20 μM, 18 h; Sigma-Aldrich) was used to induce DA neuronal damage. After neurotoxin treatment, isolated mitochondria were added to fresh media twice a day on days 7 and 8.

Retrovirus production and transduction

For dopaminergic neuronal differentiation in NPCs, *Nurr1-Mash1*-expressing retroviruses were produced from 293GPG packaging cells. DNA constructs were constructed using the pCL retroviral vector plasmid. 293GPG cells were transfected with DNA using Lipofectamine2000 (Invitrogen). The supernatant containing the viral particles was collected 72 h after transfection. The viral supernatant was added to the primary NPCs and incubated for 2 h in the presence of polybrene (2 μg/ml, Sigma-Aldrich).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). cDNA was synthesized using a Superscript kit (Invitrogen) in a final volume of 20 μl, and PCR was performed using the following primers and conditions: *Tuj1* (forward: 5′-TGCGTGTGTACAGGTGAA-3′, reverse: 5′-AGGCTGCATAGTCATTTC-3′; 60°C, 30 cycles, 200 bp) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; forward: 5′-GGCATTGCTCTCATTGACAA-3′, reverse: 5′-AGGGCCTCT CTCTTGCTCTC-3′, 60°C, 23 cycles, 165 bp).

Stereotaxic injection in the animal model of ischemic brain injury

All animal experiments were performed according to the IACUC guidelines (2020-0238A). Male SD rats were housed in an animal facility under specific pathogen-free conditions with a 12-h light/dark cycle. To generate the MCAO model, 10-week-old male SD rats (280-320 g) were anesthetized using 5% isoflurane in 70% N_2O and 30% O_2. The body temperature of the rats was maintained at 36.5 ± 0.5°C during surgery using a heating pad (Harvard Apparatus, Holliston, MA, USA). The anesthetized rat was placed in the supine position, and an incision was made in the midline of the neck to expose the right external carotid artery (ECA). The right ECA was ligated with a thread. After fixing the common carotid artery (CCA) and internal carotid artery (ICA), the ECA was incised using microscissors. Subsequently, 2.5 cm of 4-0 nylon was inserted into the ICA, and the CCA was occluded using a clip. After 1 h, the

clip and the inserted suture were removed for reperfusion. To perform stereotaxic injections, the bregma of anesthetized rats was exposed. The injection site (4 mm lateral to the bregma) was drilled using a saline (0.89% NaCl) drip. Ten microliters of the sample were stereotaxically injected into the striatum over 10 min using a 26-gauge Hamilton syringe (80330; Hamilton, Reno, NV, USA) and a microinjector.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich). After 15-20 min, the cells were washed thrice with 0.1% bovine serum albumin/PBS (BSA/PBS) wash buffer and blocked for 1 h using 0.3% Triton X-100 (Sigma-Aldrich) and 10% normal goat serum (NGS; Invitrogen). After blocking, the cells were incubated overnight with primary antibodies. The following primary antibodies were used: glial fibrillary acidic protein (GFAP; 1:2000; DAKO, Glostrup, Denmark), TUJ1 (1:2000; Covance, Richmond, CA, USA), and TH (1:2000; Pel-Freez, Rogers, AR, USA). The secondary antibodies used for visualization were Alexa Fluor 488 (1:500; Thermo Fisher Scientific) and rhodamine (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The nuclei were stained with VECTASHIELD with 4′,6-diamidino-2-phenylindole mounting medium (Vector Laboratories, Burlingame, CA, USA), and photographs were obtained using an epifluorescence microscope (LEICA Microsystem, Wetzler, Germany).

2,3,5-triphenyltetrazolium chloride (TTC) staining

The brains of MCAO rats were harvested 24 h after ischemia-reperfusion. The brains were sectioned into five 2-mm-thick slices using a brain matrix. The slices were stained with a 2% TTC (Sigma-Aldrich) solution at 37°C for 10 min. The stained samples were fixed in 4% paraformaldehyde. The infarct volume was measured using Image J 1.42 software (NIH, Bethesda, MD, USA). The infarct volume was calculated as follows: infarct volume (%) = (infarct area × thickness of each slice)/(total area × thickness of each slice) × 100.

Cell counting and statistical analysis

Cell counting was performed using a uniform random selection of 5-10 microscopic fields per well, with 3-4 wells per experimental condition. All experiments were performed at least thrice to identify statistical significance. Data are expressed as mean ± standard error (S.E.). A paired t-test was performed using SigmaPlot for Windows (version 10.0; Systat Software GmbH, Erkrath, Germany) when more than two groups were compared.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time as the data is a part of an ongoing study.

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