Mitochondrial Transplantation Improves Stroke-induced Brain Injury: Possible Involvement of Selective Component Recombination

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Research

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Abstract

Background: Ischemic stroke results in high morbidity and mortality, and mitochondrial dysfunctions play a crucial role in the associated pathological process. Although exogenous mitochondria were used to treat ischemic stroke-induced brain injury, its effects and related mechanisms remain poorly understood, as is the fate of exogenous mitochondria during/after internalization by targeted cells.

Methods: The mitochondrial morphology, membrane potential, DNA copy number, mitochondrial stress, metabolic characteristics and tumorigenicity of mNSCs and Neuro-2a cells were evaluated. Hypoxia/reoxygenation-induced cell injury was performed, and after mitochondrial supplementation, the viability, ROS levels, apoptosis and transcriptomic changes were assessed by CCK-8, DCHF-DA probes, flow cytometry, WB and next-generation sequencing analyses. The fate of exogenous mitochondria was further explored using fluorescent dyes and fusion protein analyses during/after internalization by targeted cells. Rat tMCAO models were generated using a suture-occluded method, and at 24 h after mitochondrial transplantation, behavioral changes and brain infarction areas were estimated by multiple score scales and TTC staining, respectively.

Results: In this research, we found that mitochondria of Neuro-2a cells had some notable differences compared to that of mNSCs on mitochondrial membrane potential, DNA copy number, stress response and metabolic characteristics, but their shapes were similar and were both no tumorigenicity. Exogenous mitochondrial treatment could increase the cellular viability in an oxygen-dependent pattern, decrease the cellular ROS generation and apoptosis, and alter the transcriptomic characteristics after subjected to hypoxia/reoxygenation in vitro. Selective component recombination might occur during/after internalization of exogenous mitochondria by host cells and was observed with mitochondrial fluorescent dyes and engineered fusion protein. Moreover, mitochondrial transplantation could significantly improve tMCAO-induced rat neurobehavioral deficiency and brain infarction.

Conclusions: The results of our present study offer a promising therapeutic strategy for ischemia/reperfusion-induced brain injury and provide preliminary insights regarding the effects and fate of exogenous mitochondria during/after being internalized into host cells.

Background

Stroke is a type of acute cerebrovascular disease that is commonly divided into ischemic and hemorrhagic strokes and is considered to be one of the primary causes of human death and disability worldwide[1-4]. The mean global lifetime risk of stroke for adult humans (≥ 25 years old) is 24.9 % (men: 24.7 %; women: 25.1 %), and the related risk for ischemic stroke is 18.3 %[1, 2]. Ischemic stroke accounts for over 80 % of all stroke patients and is often triggered through a sudden occlusion of brain arteries[3, 5]. After suffering ischemic stroke, brain tissue injury induced by an interruption of blood supply for specific cerebral regions, which results in the loss of glucose and oxygen to brain tissue, can rapidly cause neurological dysfunctions[3, 6-9]. Furthermore, following blood reperfusion, damaged brain tissues

can be further harmed by the excessive production of reactive oxygen species (ROS) due to the restoration of oxygen and nutrients to damaged brain cells, causing a so-called ischemia-reperfusion (I/R) injury[3, 6-9]. Major contributors to the pathological process of ischemic stroke include the overproduction of ROS, dramatically increased extracellular glutamate levels, and the potent activation of neuroinflammation responses[6, 7, 9]. Among different impairments, the dysfunctions of mitochondria within brain cells, especially in neurons, play an extremely crucial role in the pathophysiological process of ischemic stroke[3, 9]. Damage to mitochondrial function can lead to a lack of cellular energy, which is indispensable for normal cellular activities, resulting in the death of a large number of brain cells (apoptosis and/or necrosis)[3, 9]. Currently, primary strategies to improve ischemic stroke-mediated brain injury include the recanalization of blocked brain blood vessels by thrombectomy and/or thrombolysis[4, 8, 10]. In addition, antioxidants, anticoagulants, and neuroprotective agents are also highly promising supplements for ischemic stroke therapy. Remarkable progress has been achieved for the treatment of this disease, yet for the secondary brain injury induced by reperfusion, there are no well-acknowledged therapeutics or methods that can produce significant therapeutic effects. Studies have shown that mitochondria within cells not only act as energy factories to generate sufficient energy supply but are also involved in a wide variety of the physiological activities of cells, including calcium homeostasis, ROS production, hormone biosynthesis and cellular differentiation[3, 9, 11]. Recently, the transplantation of exogenous mitochondria has been intensively investigated as a therapeutic strategy and has demonstrated beneficial effects for various kinds types human disorders, including I/R injury (acute lung injury (ALI)[12], liver injury[13, 14], heart injury[15-19] and limb injury[20]), acetaminophen-induced liver disorder[21], non-alcoholic fatty liver disease[22], breast cancer[23-25], and lung diseases (pulmonary artery hypertension[26], hyperresponsiveness of the airway [27]). Furthermore, the transplantation of exogenous mitochondria has been reported to be capable of improving multiple central nervous system (CNS) disorders[28, 29] such as stroke[30, 31], spinal cord injury (SCI)[32, 33], schizophrenia[34], depression-like behaviors[35] and neurodegenerative diseases such as Parkinson's disease (PD)[36, 37]. In addition, Kuo and colleagues have also reported that the direct local injection of exogenous mitochondria could prevent axonal degeneration induced by the crushing of the sciatic nerve[38]. Furthermore, Emani et al. have also shown a promising clinical application based on mitochondrial autotransplantation treatment[39]. Thus, mitochondrial transplantation holds enormous therapeutic potential for various CNS diseases that involve mitochondrial dysfunction, especially for ischemic stroke. However, for mitochondrial transplantation-based therapy, a number of factors remain to be poorly investigated, including the optimal source of mitochondria, fate tracking within host cells, the mechanism of action, and the safety and effectiveness of therapeutic mitochondria, especially in ischemic stroke-induced brain injury,. Therefore, in our present study, we first compared the relevant characteristics of mouse neural stem cells (mNSCs) and Neuro-2a cells as potential sources of mitochondria for transplantation. Subsequently, we used Neuro-2a cell-derived mitochondria to investigate their protective effects in hypoxia-reoxygenation (H/R)-induced cell injury and the associated mechanisms as well as their fate within host cells during/after internalization in vitro. Finally, we further assessed the potential effects of transplanted mitochondria from Neuro-2a cells in tMCAO-induced rat brain injury models.

Methods

Materials and reagents

Neuro-2a cells (SCSP-5035) and U-87 MG cells (TCHu138) were purchased from the cell bank of the Typical Culture Preservation Committee, Chinese Academy of Sciences (CAS). Human 293T and Huh7 cells were kindly provided by Dr. Gao Liu (Department of Liver Surgery, Zhongshan Hospital, Shanghai Medical College, Fudan University). Dulbecco's modified eagle medium (DMEM)/F-12 (Cat. No. 11330032), fetal bovine serum (FBS) (Cat. No. 10099141C), N-2 (Cat. No. 17502048), B-27 (Cat. No. 17504044), L-glutamine (Cat. No. 25030081), penicillin-streptomycin (P/S) (Cat. No. 15140122) were purchased from Gibco (Thermo Fisher Scientific, USA). Hoechst 33342 (Cat. No. H3570, Thermo Fisher Scientific, USA) was used to stain the cell nuclei. Epidermal growth factor (EGF) (Cat. No. AF-100-15) and basic fibroblast growth factor (bFGF) (Cat. No. AF-100-18B) were purchased from PeproTech (Cranbury, NJ, USA). Alexa Fluor[®] 647 mouse anti-nestin (Cat. No. 560393, 1:1000) and Alexa Fluor[®] 488 mouse anti-Sox2 (Cat. No. 560301) antibodies were from purchased BD Biosciences (NJ, USA). A mitochondrial membrane potential assay kit with JC-1 (Cat. No. C2006) and DAPI (Cat. No. C1005) was obtained from Beyotime (Shanghai, China). 2,3,5-Triphenyl tetrazolium chloride (TTC) was purchased from Adamas (Cat. No. 01057376, Shanghai, China). Silicon-coated monofilament sutures for rat tMCAO (Cat. No. L3800/L3600) were purchased from Guangzhou Jialing Biotechnology (Guangdong, China).

Experimental animals

Neonatal C57BL/6 mice were purchased from Shanghai Lingchang Biotechnology Co., Ltd. (Shanghai, China). Adult male Sprague-Dawley rats (7-8 weeks old, 250-300 g) were obtained from Shanghai Super-B&K Laboratory Animal Corp. Ltd. (Shanghai, China). All experimental procedures and animal care were approved by the Animal Welfare and Ethics Group, Laboratory Animal Science Department, Fudan University and were carried out according to the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health. All rats were housed in a controlled environment with appropriate light (light/darkness: 12 h), temperature (18-26 °C) and humidity (50-70 %) conditions. All animals were provided a standard diet and water during the study. Experimental rats were grouped using the random number table method.

Cell culture

After being successfully isolated from the brains of neonatal mice (day 1), mNSCs were cultured in FBS-free DMEM/F-12 medium supplemented with 2 % (v/v) B27, 1 % (v/v) N-2, EGF (20 ng/mL), recombinant human bFGF (20 ng/mL), L-glutamine (2 mM) and P/S (P: 100 U/mL; S: 100 mg/mL). For adherent cell cultivation, mNSCs were cultured in plates, dishes or flasks precoated with Matrigel (Cat. No. 354277, Corning, NY, USA). The mNSCs were passaged and used upon reaching 80-90 % confluency. The mouse Neuro-2a cells, SV40T antigen-expressing human 293T cells, U-87 MG cells and human Huh7 cells were cultured in high-glucose DMEM supplemented with 10 % (v/v) heat-inactivated FBS and P/S (P: 100

U/mL; S: 100 mg/mL). After reaching 70-90 % confluency, the cultured cells were passaged and used. All cells were cultured in an incubator at 37 °C under an atmosphere with 5 % CO₂.

Immunofluorescence (IF)

Briefly, the medium of cultured cells was discarded, and the cells were washed with phosphate buffer saline (PBS) 3 times. Then, the cells were fixed with fixation buffer (Biolegend) for 20 min, washed with PBS, and then incubated with membrane-rupturing reagents for another 20 min. Subsequently, after being washed with PBS, the prepared cells were coincubated with specific primary antibodies for 1 h. Then, after being counterstained with 4',6-diamidino-2-phenylindole (DAPI) for approximately 10 min, cell samples were observed and imaged with a confocal microscope (Nikon) or directly detected by flow cytometry analysis without nuclear staining. To assess mitochondrial colocalization with lysosomes, mitochondria from 293T cells expressing COX8A N-terminal signal peptide-mCherry fusion protein were isolated and incubated with a culture of normal 293T cells for 24 h, after which the cells were washed twice with PBS. Next, the cells were incubated with Lyso Dye (Cat. No. MD01, Dojindo Laboratories, Kumamoto, Japan) at 37°C for 30 min. Then, after being counterstained with Hoechst 33342 at 37 °C for 10 min, the cells were observed and imaged with a confocal microscope (Nikon). All experimental procedures were conducted in darkness at room temperature (RT).

Transmission electron microscopy (TEM)

To observe the morphological characteristics of Neuro-2a cells and mNSCs, TEM was performed as described in previous studies[31, 32]. Briefly, cultured Neuro-2a cells and mNSCs were fixed with 2.5 % glutaraldehyde (2 h, RT) and then centrifuged (300 × g, 5 min). Subsequently, the harvested cells were postfixated with precooled 1 % osmic acid (2 h, 4 °C) and then centrifuged again (300 × g, 5 min). After gradient alcohol dehydration and penetration with a solution of acetone and epoxy resin at different proportions, the cell samples further embedded into epoxy resin and solidified for 48 h. Subsequently, the embedded samples were sectioned (thickness: 60-100 nm) and then double-stained with 3 % uranyl acetate and lead citrate. Finally, the stained sections were observed and imaged by TEM (Tecnai G2 20 TWIN®FEI Company, Oregon, USA).

Mitochondrial labeling

The mitochondrial fluorescent dyes MitoTracker™ Red CMXRos (Cat. No. M7512, Thermo Fisher Scientific, Waltham, MA, USA), MitoBright Deep Red (Cat. No. MT12, Dojindo Laboratories, Kumamoto, Japan) and MitoTracker™ Green FM (Cat. No. M7514, Thermo Fisher Scientific, Waltham, MA, USA) were used to mark Neuro-2a cells or mNSCs, respectively, by coincubation at 37 °C for 45 min in darkness, after which the cells were washed 3 times with PBS and the supernatants were discarded. Next, the cells were counterstained with Hoechst 33342 (Cat. No. H3570, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min, washed 3 times with PBS and then observed and imaged with a confocal microscope (Nikon). In addition, COX8A gene N-terminal signal peptide-mCherry fusion protein-expressing pLV-Mito-mCherry lentiviral vector (Cat. No. VL3512, Inovogen Tech. Co., Ltd., Chongqing, China) was used to transfect

target cells (293T cells, MOI: 30) and mark mitochondria within cells. Subsequently, marked mitochondria were isolated from labeled or transfected cells for further experiments.

Isolation of mitochondria

Appropriate mitochondria from mNSCs and Neuro-2a cells were isolated using a Mitochondria Isolation kit for Cultured Cells (Cat. No. #89874, Thermo Fisher Scientific, USA) as previously described[32, 40] with minor modifications. Briefly, after the cultured cells were digested (trypsin) and centrifuged ($300 \times g$, 5 min), the supernatant was removed and the cells were resuspended in mitochondria isolation reagent A (800 μ L) in a 2.0-mL microcentrifuge tube before being vortexed for 5 s and then incubated for 2 min on ice. Then, reagent B (10 μ L) was added and the sample was incubated in situ for 5 min. The sample was then vortexed at maximum speed 5 times (1 min each time) and then mixed with reagent C (800 μ L). Subsequently, the mixture was centrifuged ($700 \times g$, 10 min, 4 °C), and the resulting supernatant was further centrifuged ($12000 \times g$, 15 min, 4 °C). Finally, fresh mitochondria were obtained and used for subsequent experiments.

Mitochondrial membrane potential (MMP) analysis

The MMP of cultured cells was assessed using a Mitochondrial Membrane Potential Assay kit with JC-1 (Cat. No. C2006, Beyotime Biotechnology, Shanghai, China) based on a previously described method[41, 42]. Single-cell suspensions of mNSCs and Neuro-2a cells were prepared and then coincubated with JC-1 working solution for 20 min at 37 °C. Subsequently, sample cells were centrifuged ($600 \times g$, 4 °C, 5 min) and then washed with JC-1 buffer solution 2 times before being resuspended and assayed by flow cytometry.

Polymerase chain reaction (PCR)

Absolute quantitative PCR was performed as previously described with some modifications[43-45]. Briefly, genomic DNA (gDNA) from mNSCs and Neuro-2a cells was extracted using a HiPure Blood DNA Mini kit (Cat. No. D3111-03, Guangzhou Magen Biotechnology Co. Ltd., Guangdong, China) according to the manufacturer's directions. The genes mt-ND1 and mt-RNR1 were used to assess mitochondrial DNA (mtDNA) levels, while the genes β -globin and β -actin were used to evaluate the nuclear DNA content. The sequences of the primers used to analyze these genes are described in detail in Table 1S. Then, the obtained gDNA was PCR amplified, detected by agarose gel electrophoresis and used to perform TA cloning. Next, plasmids were extracted from the positive colonies, and appropriate standards for absolute quantitation through quantitative PCR analysis were obtained. Then, the acquired standards were serially diluted to generate a standard curve. All DNA samples were analyzed by quantitative PCR using AceQ qPCR SYBR Green Master Mix (Cat. No. Q111-03, Vazyme Biotech Co., Ltd., Jiangsu, China) in a Real-time PCR Instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the copy numbers of mt-ND1, mt-RNR1, β -globin and β -actin were determined with the calculated CT values and standard curves. Finally, the relative abundance of mitochondria was reported as the ratio of mtDNA to nuclear DNA (mt-ND1/ β -globin, mt-RNR1/ β -actin). The experiment was repeated 3 times.

Mitochondrial stress test of cultured cells

Mitochondrial stress tests were performed using the Seahorse XF analysis platform according to previous methods[33, 45, 46]. Briefly, Neuro-2a cells and mNSCs were seeded onto 96-well XF-96 plates (Seahorse Biosciences, Billerica, MA, USA) precoated with Matrigel. The oxygen consumption rate (OCR) values of cultured cells were measured using a Seahorse XFe-96 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, MA, USA) for untreated cells (basic OCR) or following the addition of mitochondrial respiration inhibitors to the system. The ATP synthase inhibitor oligomycin (10 μ M), the oxidative phosphorylation uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 10 μ M) and the electron transport chain inhibitor rotenone/antimycin (5 μ M) were added to the system to assess mitochondrial oxidative respiration activity through OCR measurements. The obtained OCR values were normalized for total protein content per well.

Metabolomic analysis

Metabolomic analysis was performed using the liquid chromatography-mass spectrometry (LC-MS) method as described in previous reports [47, 48]. mNSCs and Neuro-2a cells (n = 8) were seeded onto the petri dishes (10 cm) and cultured for 24 h, harvested upon reaching 90 % confluency and then resuspended in 1 mL of a precooled chromatographic grade methanol-acetonitrile-water solution. Then, the samples were vortexed for 1 min, lysed by ultrasonication (30 min, 2 times) in an ice water bath and then incubated for 1 h at -20 °C. After being centrifuged (14000 $\times$ g, 4 °C, 20 min), the obtained cell samples were stored at -80 °C for subsequent use. Then, hydrophilic interaction liquid chromatography (HILIC) was used for LC separation of the samples with an Agilent 1290 Infinity LC ultra-performance liquid chromatography (UPLC) system (25°C, 0.3 mL/min). Then, the obtained samples were further analyzed by MS in the electrospray ionization source (ESI)-based cationic and anionic modes using a Triple TOF 5600 Mass Spectrometer (AB SCIEX, USA). Subsequently, the acquired raw LC-MS/MS data were converted to .mzXML format using ProteoWizard (ProteoWizard, Palo Alto, CA, USA) and then processed and analyzed with the XCMS package in the R software environment for peak alignment, retention time correction and peak area extraction. Next, the structures of metabolites were identified by an exact mass number matching (< 25 ppm) and secondary spectrum matching based on a self-built database. Last, the R software environment (R Foundation for Statistical Computing, Vienna, Austria) and MetaboAnalyst 4.0 online tools (<http://www.metaboanalyst.ca>) were used to perform principal component analysis (PCA), cluster analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Tumorigenicity detection

To assess the tumorigenicity of grafted mitochondria in vivo, mitochondria isolated from Neuro-2a cells and mNSCs were separately injected into the right infra-axillary of nude mice. Macroscopic images were acquired with a digital camera at 6 months after cellular or mitochondrial injection. Different types of mouse tissues (heart, liver, spleen, lung, kidney and injection site-related subcutaneous lymph node tissues) were collected after 6 months and prepared for perform hematoxylin & eosin (H&E) staining after

the transplantation of mitochondria from Neuro-2a cells or mNSCs. Briefly, prepared paraffin-embedded tissue sections were stained with eosin for 10 s and then counterstained with hematoxylin for 5 min. Following dehydration in a graded ethanol series and being cleared in xylene, the sections were mounted with neutral balsam, and images were obtained with a microscope (Leica, DM2500, Germany).

Hypoxia-reoxygenation (H/R) analysis

H/R injury of Neuro-2a cells was performed according to a previously described method with minor modifications[49]. Briefly, experimental cells were placed in a hypoxic environment (1 % O₂, 5 % CO₂ and 94 % N₂) generated using a three gas incubator for 48 h and then further cultivated under conventional conditions (reoxygenation) for 24 h. The cultured cells were divided into 3 groups: control group [conventional culture (48 h) + replacing medium + continuing conventional culture (24 h)], H/R group [hypoxic culture (48 h) + replacing medium + continuing conventional culture (24 h)] and H/R + mitochondrial treatment group [hypoxic culture (48 h) + replacing medium (containing exogenous mitochondria) + continuing conventional culture (24 h)].

CCK-8 assay

The viability of Neuro-2a cells was using a Cell Counting kit-8 (CCK-8) (Cat. No. CK04, Dojindo Laboratories, Kumamoto, Japan) as previously described[50-52]. Briefly, cultured Neuro-2a cells were incubated with CCK-8 working solution at 37 °C for 3 h in darkness. Then, the CCK-8 working solution-treated Neuro-2a cells were analyzed at 450 nm using a multifunctional microplate reader (Molecular Devices, Sunnyvale, CA, USA) to obtain optical density values.

Detection of ROS levels

DCFH-DA probes (Cat. No. S0033M, Beyotime Biotechnology, Shanghai, China) were used to measure the ROS levels in Neuro-2a cells according to the manufacturer's instructions[53, 54]. Briefly, after being incubated with DCFH-DA probes (10 µmol/L, excitation and emission wavelengths of 488 and 525 nm, respectively) at 37 °C for 30 min, Neuro-2a cells were analyzed using a multifunctional microplate reader (Molecular Devices, Sunnyvale, CA, USA) or collected by centrifugation (300 × g, 5 min). Then, after being resuspended in PBS, the DCFH-DA-labeled Neuro-2a cells were further analyzed by flow cytometry.

Western blot (WB) analysis

WB analysis was performed as previously described[53, 55]. The following primary antibodies were used for WB detection: anti-MFN1 (Cat. No. 13798-1-AP, 1:500), anti-OPA1 (Cat. No. 27733-1-AP, 1:1000) and anti-DRP1 antibodies (Cat. No. 12957-1-AP, 1:1000) were all purchased from Proteintech (Chicago, IL, USA); and anti-Bax (Cat. No. ab182733, 1:2000), anti-Bcl-2 (Cat. No. ab182858, 1:2000), anti-caspase-3 (Cat. No. ab184787, 1:2000) and anti-GAPDH antibodies (Cat. No. ab181602, 1:10000) were all purchased from Abcam (Cambridge, Cambs, UK). The anti-GAPDH antibody was used as an internal reference. Protein expression levels were detected using a Gel-Pro Analyzer (Media Cybernetics, MD, USA).

Detection of apoptosis levels

Neuro-2a cell apoptosis was evaluated using an Annexin V-FITC/PI Apoptosis Detection kit (Cat. No. 556547, BD Biosciences, Franklin Lakes, NJ, USA) as previously described[56, 57]. Briefly, prepared Neuro-2a cells were co-cultured with Annexin V-FITC dyes, after which propidium iodide was used to counterstain the treated cells. After being further cultivated for 15 min at RT in darkness, the double-stained Neuro-2a cells were detected by flow cytometry.

Transcriptomic analysis

Transcriptomic detection and analysis of cultured Neuro-2a cells was performed as previously described with some modifications[58, 59]. Total RNA was extracted from cultured Neuro-2a cells with TRIzol reagent (Cat. No. 15596018, Thermo Fisher Scientific, USA) and then dissolved into DNase/RNase-free water (Cat. No. ST876, Beyotime Biotechnology, Shanghai, China). The purity and quantity of RNA samples were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). Then, the RNA samples were used for transcriptomic analysis[57]. Subsequently, the acquired data were used for downstream analyses, including PCA and KEGG pathway enrichment analysis using the R software environment (R Foundation for Statistical Computing, Vienna, Austria). Subsequently, the data were visualized with a PCA plot, a heatmap, a Venn diagram and a bubble chart.

Rat tMCAO and mitochondrial delivery

Adult male Sprague-Dawley rats were used to induce tMCAO injury according to a previously described method with some modifications[60, 61]. In brief, after being anesthetized by an intraperitoneal injection of 2 % pentobarbital sodium (45 mg/kg), the rats were placed in a prone position. Then, after the left common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were exposed, a silicon-coated monofilament suture was gradually inserted through the left ECA and was moved up into the left ICA to successfully occlude the left middle cerebral artery (MCA) and remained in situ for 120 min. Subsequently, the suture was carefully removed, the ECA was permanently ligated, and the incision was sutured. Sham-operated rats were subjected to the same procedure except that the 120 min-occlusion of the MCA with a silicon-coated monofilament suture was not performed. Experimental animals were then placed into individual cages and provided a standard diet and water. For intravital delivery of exogenous mitochondria, the prepared mitochondrial solution (isolated mitochondria, 10 μ L) or vehicle solution (PBS, 10 μ L) was immediately injected into the ICA after reperfusion started.

Evaluation of behavioral deficits

Neurobehavioral defects were evaluated 24 h after mitochondrial transplantation using multiple rating scales, including the Clark general functional deficit score[62, 63], the Clark focal functional deficit score[62, 63], the modified neurological severity score (mNSS)[61, 64] and the Rotarod test[61, 65] as

previously described. Behavioral assessments were conducted by two skillful investigators who were both blinded to the animal groups.

TTC staining

TTC was used to evaluate the brain infarct size of tMCAO rats as previously described with minor modifications[66, 67]. Briefly, 24 h after tMCAO, the rats were deeply anesthetized and transcardially perfused with PBS to clear blood components of the brain vascular system, after which the rat brains were obtained and cut into 2-mm-thick coronal sections. Subsequently, the brain sections were incubated with a 2 % TTC solution at 37 °C for 30 min in darkness. Then, stained slices were placed from the frontal to occipital order, and macroscopic images were obtained with a digital camera. Infarct areas were calculated using Adobe Photoshop 21.0.0 (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

Data that satisfied a Gaussian distribution (Shapiro-Wilk test) and homogeneity of variance (F-test) are presented as the means $\pm$ standard deviation (SD), and Student's t-test or one-way analysis of variance (ANOVA) were used to compare the differences between two groups or among multiple groups, respectively. Data with a nonnormal distribution are presented as the medians (25 % and 75 % quantiles), and Mann-Whitney U-test was taken into consideration. Statistical analysis and diagram generation were performed using GraphPad Prism 8.0.1 (GraphPad Software, Inc., San Diego, CA, USA). A P-value < 0.05 was considered to indicate a significant difference.

Results

Morphological characteristics of mitochondria from mNSCs and Neuro-2a cells

Neonatal mouse-derived brain cells were cultured in suspension for several days until a number of spherical cell masses developed (neurosphere, a typical marker of NSCs) (Figure S1 A), after which they were further cultivated as adherent cells (Figure S1 B). The obtained cells were double-stained by both Nestin and Sox2 (two representative markers of NSCs) (Figure S1 C), demonstrating that mNSCs had been successfully obtained. To observe the morphological characteristics of mitochondria from Neuro-2a cells and mNSCs, fluorescent staining was performed, and the results suggested that the mitochondrial shapes for both cell types were primarily club-shaped and could connect to form net-like structures (Figure 1 A, B). Similarly, the TEM results showed that the two types of cells exhibited short rod-like and particle-like mitochondria shapes (Figure 1 C, D). These results demonstrated that there were no noticeable morphological differences in mitochondria between the Neuro-2a cells and mNSCs.

MMP and mitochondrial abundance for Neuro-2a cells and mNSCs

To assess the MMP status of mNSCs and Neuro-2a cells, JC-1 dyes were used flow cytometry analyses were performed. Typical images of JC-1 staining to assess MMP detection in Neuro-2a cells (Figure 1 E) and mNSCs are shown in Figure 1 F, and the statistical results suggested that the Neuro-2a cells had a

significantly higher MMP status than that of mNSCs (Neuro-2a cells vs. mNSCs: 10.55 ± 0.85 vs. 2.56 ± 0.36 , $p < 0.01$) (Figure 1 G). In addition, we also observed that mNSCs exhibited significantly higher relative abundances of mtDNA than that of Neuro-2a cells based on the observed ratio values of mt-ND1/ β -globin (mNSCs vs. Neuro-2a cells: 731.1 ± 110.4 vs. 374.0 ± 11.5 , $p < 0.01$) (Figure 1 H) and mt-RNR1/ β -actin (mNSCs vs. Neuro-2a cells: 593.4 ± 108.3 vs. 149.1 ± 13.07 , $p < 0.01$) (Figure 1 I).

Oxidative respiration capacity of mitochondria from Neuro-2a cells and mNSCs

We subsequently analyzed the oxidative respiration capacity of mitochondria from Neuro-2a cells and mNSCs based on the Seahorse XF analysis platform. The schematic diagram of the mitochondrial stress test is clearly shown in Figure 1 J. The acquired results showed that basal OCR values of Neuro-2a cells (1×10^5 cells) were significantly higher than those observed for mNSCs (1×10^5 cells) (Neuro-2a cells vs. mNSCs: 248.70 ± 56.33 pmol/min vs. 22.14 ± 5.09 pmol/min, $p < 0.01$) (Figure 1 K). Similarly, Neuro-2a cells (1×10^5 cells) exhibited higher maximal OCR values than those observed for mNSCs (1×10^5 cells) (Neuro-2a cells vs. mNSCs: 363.90 ± 123.70 pmol/min vs. 28.14 ± 7.50 pmol/min, $p < 0.01$) (Figure 1 L). These results suggested that compared to mNSCs, mitochondria from Neuro-2a cells exhibited a relatively stronger oxidative respiration capacity.

Metabolomic characteristics of Neuro-2a cells and mNSCs

Through LC-MS analysis, we successfully identified 231 and 220 metabolites under the cationic and anionic modes, respectively, from mNSCs and Neuro-2a cells. Then, the results of PCA analyses (Figure 2 A, E) and heatmaps from cluster analyses (Figure 2 B, F) indicated that mNSCs and Neuro-2a cells exhibited remarkably different expression patterns of metabolites in the cationic and anionic modes. In addition, differential analysis of expression for different metabolites based on PLS-DA suggested that under the cationic mode, compared with that observed for mNSCs, Neuro-2a cells had 115 upregulated and 37 downregulated metabolites (Figure 2 C), while in anionic mode, 108 metabolites were upregulated and 24 metabolites were downregulated (Figure 2 G). After subsequent KEGG pathway enrichment analyses, we observed that under the cationic mode, histidine metabolism, nicotinate and nicotinamide metabolism, alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism primarily contributed to the expression pattern differences for the two cell types (Figure 2 D), while alanine, aspartate and glutamate metabolism, amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism and pentose phosphate pathway were identified in the anionic mode (Figure 2 H).

Tumorigenicity detection of mitochondria from mNSCs and Neuro-2a cells

To assess the feasibility and safety of mitochondria from mNSCs and Neuro-2a cells, the tumorigenicity of grafted mitochondria in vivo was assessed. Our results indicated that after right infra-axillary injection, transplanted mitochondria from Neuro-2a cells (Figure 1 M) and mNSCs (Figure 1 N) did not cause any visible tumor formation for up to 6 months. Furthermore, no evident tumor formation was observed by H&E staining of subcutaneous lymph node tissues from injection sites (Figure 1 O, P). In addition, H&E

staining of the heart (Figure S2 A), liver (Figure S2 B), spleen (Figure S2 C), lung (Figure S2 D) and kidney (Figure S2 E) also indicated that no obvious tumorigenesis occurred after the injection of mNSCs-derived mitochondria. Similar results were obtained after the injection of mitochondria from Neuro-2a cells, with no tumor formation observed in the heart (Figure S2 F), liver (Figure S2 G), spleen (Figure S2 H), lung (Figure S2 I) and kidney tissues (Figure S2 J).

The results of our present study suggest that mitochondria from mNSCs and Neuro-2a cells have no apparent differences in morphological features but relatively notable distinctions in the metabolomic profiles. In addition, mitochondrial copy numbers of mNSCs were higher than those observed for Neuro-2a cells, whereas the MMP status and oxidative respiration capacity of mitochondria from Neuro-2a cells were stronger than that observed for mNSCs. Furthermore, as *in vivo* injection of mitochondria from these cells did not cause observable tumor formation, the transplantation of exogenous mitochondria was shown to be relatively safe and feasible. Therefore, we selected the Neuro-2a cell-derived mitochondria for use in subsequent experiments.

Effects of exogenous mitochondria on the viability of Neuro-2a cells

In the present study, we observed that after Neuro-2a cells were cultured under hypoxic conditions (1 % O₂) for 48 h, the treatment with exogenous mitochondria had different effects under different culture conditions. If exogenous mitochondria-treated Neuro-2a cells continued to be cultured under hypoxic conditions, their viability was further dramatically decreased (hypoxia vs. hypoxia + Mito: 1.00 ± 0.03 vs. 0.07 ± 0.01 , $p < 0.01$) (Figure 3 A), whereas for cells that were continuously cultured with reoxygenation, the addition of fresh mitochondria significantly increased their viability (reoxygenation vs. reoxygenation + Mito: 1.00 ± 0.12 vs. 1.24 ± 0.14 , $p < 0.01$) (Figure 3 B).

ROS levels of Neuro-2a cells were effectively decreased by mitochondrial addition

To evaluate the ROS levels of cultured cells, we assessed ROS contents using DCFH-DA probes. The results showed that compared to that observed in untreated cells, H/R treatment could obviously increase the ROS content (H/R vs. Control: 606.1 ± 23.45 vs. 173.2 ± 13.74 , $p < 0.01$) (Fig. 3 E), and the addition of fresh mitochondria could significantly inhibit the upregulation of ROS levels within cells (H/R vs. H/R + Mito: vs. 416.9 ± 31.59 , $p < 0.01$) (Fig. 3 E). Moreover, similar results were obtained by flow cytometry analysis and suggested that after H/R, the ROS levels within cells notably increased (H/R vs. control: 130.7 ± 2.80 vs. 52.03 ± 0.68 , $p < 0.01$) (Fig. 3 C, D). Interestingly, the mitochondrial treatment distinctly reduced the ROS levels of cells (H/R vs. H/R + Mito: vs. 110.4 ± 3.07 , $p < 0.01$) (Fig. 3 C, D).

Treatment with exogenous mitochondria can effectively reduce Neuro-2a cell apoptosis

The flow cytometry results suggested that after being subjected to the H/R treatment, the apoptosis ratio of Neuro-2a cells dramatically increased (H/R vs. control: 39.89 ± 0.65 % vs. 4.03 ± 0.05 %, $p < 0.01$) (Figure 3 F, G), which could significantly reduced by mitochondrial supplementation (H/R vs. Mito + H/R: vs. 24.66 ± 0.46 %, $p < 0.01$) (Figure 3 F, G). Similar results were obtained for the expression levels of

apoptosis-related proteins, which also suggested that H/R dramatically promoted the upregulation of the Bax/Bcl-2 ratio (H/R vs. control: 16.28 ± 3.82 vs. 1.00 ± 0.16 , $p < 0.01$) (Figure 3 H, I) and caspase-3 protein (H/R vs. control: 2.21 ± 0.12 vs. 1.00 ± 0.11 , $p < 0.01$) (Figure 3 H, J). Supplementation with fresh mitochondria could significantly downregulate the ratio of Bax/Bcl-2 (H/R vs. H/R + Mito: vs. 4.25 ± 0.34 , $p < 0.01$) (Figure 3 H, I) and protein levels of caspase-3 (H/R vs. H/R + Mito: vs. 1.65 ± 0.03 , $p < 0.01$) (Figure 3 H, J) in cultured cells.

Effects of grafted mitochondria on the transcriptome of Neuro-2a cells

Based on the PCA results, we observed that the control, H/R, and H/R + mitochondria Neuro-2a cells exhibited remarkable differentiation, and the associated PCA plot was drawn (Figure 4 A). In addition, enrichment analysis of the three groups of cultured cells also indicated similar results, and the corresponding heatmap was generated (Figure 4 A). Furthermore, the results further suggested that compared to that observed for control Neuro-2a cells, the mRNA levels of 14 genes were upregulated while 12 genes were downregulated in H/R-treated cells; for mitochondria-treated H/R cells, the mRNA levels of 27 genes increased while that of 98 genes decreased. Compared to that observed for H/R-treated cells, the mitochondrial treatment increased the mRNA levels of 17 genes, while that of only one gene was reduced. Differences in gene expression for the three groups of Neuro-2a cells are shown in the Venn diagram presented in Figure 4 C. After further performing KEGG pathway enrichment analysis, we observed that following intervention with extracted mitochondria, multiple cellular metabolism-related pathways of cultured cells were notably affected, especially for lipid metabolism-related molecules and pathways such as the PPAR signal pathway, insulin signal pathway, fat intake and digestion-related pathway, cholesterol metabolism, glycolysis and gluconeogenesis (Figure 4 D). These results indicated that the supplementation of exogenous mitochondria may be capable of altering the metabolic characteristics of cultured cells.

Transport mechanism and fate of exogenous mitochondria in targeted cells

Our results suggested that both red- and green-labeled mitochondria could be successfully isolated from MitoTracker™ Red CMXRos or MitoTracker™ Green FM-marked Neuro-2a cells, respectively. After being coincubated with red- or green dye-labeled Neuro-2a cells, green or red mitochondria could effectively enter into cultured cells and fuse with the endogenous mitochondria of targeted cells (Figure 5 A, B). Additionally, we also observed that red dye-labeled mitochondria from human U87 cells could be internalized into mNSCs and co-localize with endogenous green mitochondria after coincubation within several hours (Figure 5 C). Subsequently, we incubated red and green fluorescent dye-marked mitochondria from 293T cells with pink 647 dye-labeled 293T cells. The results showed that nearly all pink mitochondria overlapped with grafted green mitochondria, with a higher level of green mitochondria than that of pink mitochondria, while only a portion of pink mitochondria overlapped with grafted red mitochondria (Figure 6 A). These results indicate that apart from fusion with endogenous mitochondria, internalized mitochondria may have another fate that may be involved in selective component recombination from these mitochondria. We further observed that after genetically engineered 293T cells

overexpressing red fluorescent fusion protein COX8A-mCherry in mitochondria were incubated with green fluorescent dyes to mark mitochondria, the labeled red and green mitochondria exhibited almost completely co-localized (Figure 6 B). Moreover, mitochondria isolated from 293T cells double-labeled with COX8A-mCherry and green fluorescent dyes also exhibited almost completely co-localized (Figure 6 C). Furthermore, we ulteriorly carried out the related experiment and the results suggested that after mitochondria double-labeled by red COX8A-mCherry fusion protein and green fluorescent dyes co-cultured with Neuro-2a cells, red protein-marked mitochondria and green dyes-marked mitochondria have different internalized time, namely, on order, the green dyes-labeled component of mitochondria was earlier internalized into cells than red protein-marked component, and this also indicated that during the process of mitochondrial internalization, selective component recombination might occur (Figure 7 A, B, C, D, E). We also observed that exogenous mitochondria marked by red COX8A-mCherry fusion protein could co-localize with endogenous lysosomes of 293T cells after coincubation (Figure 8 A), indicating that exogenous mitochondria may be digested by lysosomes after being internalized into target cells.

Effects of mitochondrial treatment on mitochondrial dynamics

We further assessed the expression levels of mitochondrial dynamic-related proteins by WB analysis. Our results suggested that after H/R treatment, the expression levels of the mitochondrial fusion-related proteins MFN1 (control vs. H/R, $p < 0.01$) (Figure 8 B, C) and OPA1 (control vs. H/R, $p < 0.01$) (Figure 8 B, D) were dramatically reduced, while mitochondrial treatment could significantly inhibit this downregulation for MFN1 (H/R vs. H/R + Mito, $p < 0.01$) (Figure 8 B, C) and OPA1 (H/R vs. H/R + Mito, $p < 0.01$) (Figure 8 B, D). For mitochondrial fission-related protein DRP1, H/R upregulated DRP1 expression compared with that observed in the control group (control vs. H/R, $p < 0.01$) (Figure 8 B, E), while mitochondrial treatment could not notably alter DRP1 expression levels (H/R vs. H/R + Mito, $p > 0.05$) (Figure 8 B, E).

Transplantation of exogenous mitochondria could markedly improve tMCAO-induced neurobehavioral deficits of rats

Our results suggested that red fluorescent protein-labeled mitochondria from Neuro-2a cells could be successfully detected in the tMCAO-induced brain injury area after transplantation through ICA injection (Figure 9 E). To assess tMCAO-induced neurobehavioral deficiency, Clark general/focal functional deficit scores and mNSS and Rotarod tests were performed 24 h after mitochondrial transplantation. Compared with that observed in the sham group, tMCAO rats exhibited marked behavioral defects, as indicated by increased Clark general ($p < 0.05$) (Figure 9 A)/focal ($p < 0.05$) (Figure 9 B) functional deficit scores and mNSS scores ($p < 0.05$) (Figure 9 C), while transplantation of exogenous mitochondria could significantly improve the neurobehavioral deficiencies of tMCAO rats ($p < 0.05$) (Figure 9 B, C, D). In addition, the results also showed that after suffering from tMCAO, the rotation time of rats notably decreased ($p < 0.05$) (Figure 9 E), while mitochondrial transplantation could significantly reverse this reduction ($p < 0.05$) (Figure 9 E).

Mitochondrial transplantation can reduce tMCAO-induced brain infarction

To further assess the effects of grafted mitochondria on the brain infarction area of rats induced by tMCAO, MTT staining was performed 24 h after mitochondrial transplantation. Typical macroscopic images are presented in Figure 9 F, and the statistical results suggested that mitochondrial transplantation could significantly reduce the infarction area of rat brains (tMCAO vs. tMCAO + Mito: $p < 0.05$) (Figure 9 G).

Discussion

In our present study, we first compared the morphological and functional characteristics of mitochondria from Neuro-2a cells and mNSCs and observed that mitochondria from two types of cells had similar shape features. In addition, we also noted that mNSC-derived mitochondria had higher mitochondrial copy numbers than Neuro-2a cells, while mitochondria from Neuro-2a cells had stronger MMP status and oxidative respiration capacity. Moreover, we also observed that the two types of cell-derived mitochondria had markedly different metabolomic characteristics, and both types elicited no obvious tumorigenicity after being transplanted into organisms for up to 6 months. Then, mitochondria from Neuro-2a cells were selected to perform subsequent experiments. Our results suggested that after Neuro-2a cells cultured under hypoxic conditions for 48 h, supplementation with exogenous mitochondria could effectively enhance their viability, reduce ROS levels and cellular apoptosis, and alter their transcriptomic characteristics. Subsequently, we further investigated the fate of internalized mitochondria and observed that selective component recombination of mitochondria may occur during mitochondrial internalization into targeted cells and that the introduced mitochondria may fuse with endogenous mitochondria, with colocalization of internalized exogenous mitochondria and endogenous lysosomes also observed. Finally, our results also suggested that exogenous mitochondria delivered intra-arterially could successfully arrive at tMCAO-induced brain injury regions and further demonstrated that mitochondrial transplantation could reduce the neurobehavioral deficits and brain infarction area-induced by tMCAO in rats.

Mitochondria are well-known double-membrane organelles that exist in nearly all eukaryotic cells that are generally considered to be the descendants of a common ancestral organelle resulting from the integration of an endosymbiotic alphaproteobacterium (Asgard Archaea) and a host cell[68] such that mitochondria have their own hereditary material (mtDNA). Mitochondria were initially shown to function as the major energy supplier (powerhouse) of eukaryotic cells through the oxidative phosphorylation-mediated production of adenosine triphosphate (ATP), although more crucial physiological functions of mitochondria were gradually established, such as cellular metabolism[69], calcium homeostasis[70], and immunity[71,72]. Mitochondrial dysfunctions can contribute to the formation of neuroinflammation and oxidative stress, which play crucial roles in the pathophysiological processes of multiple CNS disorders, leading mitochondria to become a highly promising target for neuroprotection through the restoration of dysfunctional mitochondria and/or transplantation of normally functional mitochondria[69]. Moreover, mitochondrial dysfunctions also play a key role in various modalities of cellular death, including apoptosis, necroptosis, pyroptosis and ferroptosis[68]. Therefore, dysfunctional mitochondria are able to promote the initiation and progression of multiple CNS disorders, including ischemic stroke[70], SCI[71],

and neurodegenerative diseases[72], the latter of which include Parkinson's disease[72-74], Alzheimer's disease (AD)[72, 74, 75], amyotrophic lateral sclerosis (ALS)[72, 74] and multiple sclerosis (MS)[72, 74].

The intercellular transfer of mitochondria has been demonstrated by a number of studies. Furthermore, stem cells transplantation has been shown to reduce different damages by the transfer of mitochondria into targeted cells in injured tissues. For ischemic stroke, Hayakawa and colleagues demonstrated that functional mitochondria could be released by resident astrocytes and enter into adjacent neurons in injured brain tissue, possibly by CD38/cyclic adenosine diphosphate (ADP) ribose signaling, improving neurological outcomes[51]. Therefore, the transplantation of exogenous mitochondria may be an excellent option to treat ischemic stroke-induced brain injury. Subsequently, the results from different groups[30, 31] suggested that the replenishment of exogenous mitochondria can significantly alleviate tMCAO-induced brain injury, as indicated by improved neurological outcomes and brain infarct volume due to the reduction of oxidative stress (OS), reactive astrogliosis, apoptosis and the promotion of neurogenesis. In our present study, we obtained comparable results in that the delivery of Neuro-2a cell-derived mitochondria could significantly improve tMCAO-induced neurological deficits and brain infarction size. Generally, mitochondrial transplantation holds enormous potential for the reversion of different types of CNS disorders. However, the results of some studies have suggested that extracellular mitochondria play unfavorable roles. Mitochondria from traumatized brain tissues were able to stimulate platelets and promote their procoagulant activity, contributing to TBI-induced coagulopathy and inflammation[76]. Furthermore, in our present study, we also surprisingly observed that the protective effects of isolated mitochondria were oxygen-dependent, where the treatment of isolated mitochondria could cause detrimental effects under hypoxic conditions and promote protective effects in a normal oxygen environment. This is a novel discovery for the treatment of mitochondrial transplantation and demonstrates the potential of this approach. The normal physiological functions of mitochondria within cells are indispensable, including the production of ATP, and if mitochondria are replenished without oxygen supply restoration, these introduced mitochondria remain in a low oxygen status and can aggravate the original injury. Therefore, this offered us a reminder when we use isolated mitochondria to treat tissue injury.

Regarding intracellular trafficking and the fate of internalized mitochondria, King and colleagues demonstrated that after isolated human mitochondria were injected into 143BTK-6TG and HT1080-6TG cells for 6-10 weeks, the introduced mtDNA could replace nearly all endogenous mtDNA of the host cells, indicating that the internalized exogenous mitochondria could fuse with endogenous mitochondria and result in the complete replacement of mtDNA[77]. In our present study, we obtained similar results and showed the colocalization of isolated mitochondria and endogenous mitochondria from targeted cells. Cowan et al. reported that after being endocytosed into human induced pluripotent stem cell-derived cardiomyocytes and primary human cardiac fibroblasts by co-culture, the majority of these internalized mitochondria could effectively fuse with endogenous mitochondria within cardiac cells, although some were degraded by the endolysosomal system (early endosome, late endosomes and lysosomes) [78]. These findings are also in line with the results of our present study. Thus, fusion with endogenous

mitochondria and degradation by the endolysosomal system within host cells may represent two different fates of exogenous mitochondria.

Conclusions

Taken together, the results of the present study showed that Neuro-2a cell-derived mitochondria have some differences compared to mNSCs. However, their shapes were similar and neither exhibited tumorigenicity, and mitochondrial transplantation could reduce stroke-induced neurological defects and brain infarction size. The results of our present study provide a promising reference for the investigation and identification of sources of transplanted mitochondria as well as the fate and associated mechanisms of internalized mitochondria and their neuroprotective effects against stroke-induced brain injury.

Abbreviations

ROS: reactive oxygen species; I/R: ischemia-reperfusion; ALI: acute lung injury; CNS: central nervous system; SCI: spinal cord injury; PD: Parkinson's disease; mNSCs: mouse neural stem cells; H/R: hypoxia-reoxygenation; tMCAO: transient middle cerebral artery occlusion; CAS: Chinese Academy of Sciences; DMEM: Dulbecco's modified eagle medium; FBS: fetal bovine serum; P/S: penicillin-streptomycin; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; TTC: 2,3,5-triphenyltetrazolium chloride; IF: immunofluorescence; PBS: phosphate buffer solution; DAPI: 4',6-diamidino-2-phenylindole; RT: room temperature; TEM: transmission electron microscopy; MMP: mitochondrial membrane potential; PCR: polymerase chain reaction; gDNA: genomic DNA; mtDNA: mitochondrial DNA; OCR: oxygen consumption rate; FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; LC-MS: liquid chromatography-mass spectrometry; HILIC: hydrophilic interaction liquid chromatography; UPLC: ultra-performance liquid chromatography; ESI: electrospray ionization source; PCA: principal component analysis; PLS-DA: partial least squares-discrimination analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; VIP: variable importance in projection; H&E: hematoxylin & eosin; H/R: hypoxia-reoxygenation; CCK-8: cell counting kit-8; WB: Western blot; ECA: external carotid artery; ICA: internal carotid artery; MCA: middle cerebral artery; mNSS: modified neurological severity score; SD: standard deviation; ANOVA: analysis of variance; ATP: adenosine triphosphate; AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; MS: multiple sclerosis; ADP: adenosine diphosphate; OS: oxidative stress.

Declarations

Ethics approval and consent to participate

All experimental procedures and animal care were approved by the Animal Welfare and Ethics Group, Laboratory Animal Science Department, Fudan University and were carried out according to the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

JHZ, QX and JZ proposed the idea and conceived this study. QX and JZ conducted related experiments. QX, JZ and FX performed the data analyses. QX and JZ wrote the manuscript. JHZ, RX, FX, YTZ, TWL, JWR, KZC and QZ provided the support of experimental techniques, assistances at different stages of study and revision of the manuscript. All authors have read and consented to the final version of this manuscript.

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Figures

Figure 1

Comparison of mitochondrial characteristics from Neuro-2a cells and mNSCs and detection of tumorigenicity Morphological observations of mitochondria of Neuro-2a cells and mNSCs were performed by staining of fluorescent dyes and TEM detection. Typical fluorescent images and TEM images of Neuro-2a cells (A, C) and mNSCs (B, D) were respectively presented. MMP status of Neuro-2a cells (E) and mNSCs (F) was shown by representative fluorescent images. After further detected with flow

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Comparison of metabolomic characteristics of Neuro-2a cells and mNSCs Metabolomic data of Neuro-2a cells and mNSCs were acquired by the LC-MS method. PCA analysis, cluster analysis, difference metabolite analysis and KEGG pathway enrichment analysis were performed under cationic mode (upper plane) or anionic mode (lower plane), respectively. PCA plots (A) (horizontal axis: PC1, 74.6 % variation of entire data; longitudinal axis: PC2, 8.2 % variation of entire data) and (E) (horizontal axis: PC1, 79.5 %; longitudinal axis: PC2, 10.2 %). Cluster analysis of the top one hundred metabolites and related

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The effects of mitochondrial supplement on the cell viability, ROS production and apoptosis of Neuro-2a cells after suffered from H/R treatment At 24 h after the addition of exogenous mitochondria, CCK-8 was used to assess the cell viability of Neuro-2a cells and relatively quantitative analyses in the condition of oxygen restoration (A) and in that of hypoxic oxygen (B). ROS levels within Neuro-2a cells were detected with DCFH-DA probes and quantified with a multifunctional microplate reader (C). Subsequently, cell

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Figure 5

Exogenous mitochondria were able to co-localize with endogenous mitochondria after internalized into different types of cells by co-incubation Mitochondria of cultured cells were labeled by MitoTracker™ Green FM (green mitochondria) or MitoTracker™ Red CMXRos (red mitochondria). Representatively microscopical images were obtained. Neuro-2a cells-derived green mitochondria could co-localize with red mitochondria of Neuro-2a cells after internalization (A). Red mitochondria from Neuro-2a cells could co-localize with green mitochondria of Neuro-2a cells after co-incubation (B). Besides, human U-87 cells-

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Double-labeled mitochondria exhibited discrepant co-localization patterns after being internalized into targeted cells by co-incubation. Mitochondria of human 293T cells were co-marked by genetically engineered expression of COX8A gene N-terminal signal peptide-mCherry fusion protein (red) and MitoTracker™ Green FM (green). Typically microscopical co-localization images of red and green mitochondria within 293T cells were obtained (A). Double-labeled mitochondria were isolated from

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Figure 9

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