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Intravenous administration of mitochondria for treating experimental Parkinson's disease

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ABSTRACT

Mitochondrial dysfunction is associated with a large number of human diseases, including neurological and muscular degeneration, cardiovascular disorders, obesity, diabetes, aging and rare mitochondrial diseases. Replacement of dysfunctional mitochondria with functional exogenous mitochondria is proposed as a general principle to treat these diseases. Here we found that mitochondria isolated from human hepatoma cell could naturally enter human neuroblastoma SH-SY5Y cell line, and when the mitochondria were intravenously injected into mice, all of the mice were survived and no obvious abnormality appeared. The results of *in vivo* distribution suggested that the exogenous mitochondria distributed in various tissues including brain, liver, kidney, muscle and heart, which would benefit for multi-systemically mitochondrial diseases. In normal mice, mitochondrial supplement improved their endurance by increase of energy production in forced swimming test; and in experimental Parkinson's disease (PD) model mice induced by respiratory chain inhibitor MPTP, mitochondrial replacement prevented experimental PD progress through increasing the activity of electron transport chain, decreasing reactive oxygen species level, and preventing cell apoptosis and necrosis. Since effective drugs remain elusive to date for mitochondrial diseases, the strategy of mitochondrial replacement would provide an essential and innovative approach as mitochondrial therapy.

1. Introduction

Mitochondrial dysfunction is associated with a large number of human diseases, including neurological and muscular degeneration, cardiovascular disorders, obesity, diabetes, and rare mitochondrial diseases (Chaturvedi and Flint Beal, 2013; McFarland et al., 2007; Poole et al., 2015). Failure to produce an adequate amount of ATP or energy is considered as one of main reasons for most of mitochondrial pathologies. Moreover, mitochondrial dysfunction usually affects any organ of the body, and the clinical presentations are especially severe in high energy demanding tissues, such as central nervous system and skeletal muscle (Govindaraj et al., 2011). However, there is no cure for mitochondrial disorders to date (Codier and Codier, 2014; Suomalainen, 2011).

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. Substantial evidences have indicated that mitochondrial dysfunction, induced by mitochondrial DNA mutation and/or mitochondrial membrane rupture, plays a central role in the pathogenesis of PD (Brauer et al., 2015; Mäkelä et al., 2016). The mitochondrial dysfunction can lead to a succession of disastrous events including bioenergetic defects, oxidative stress injury, calcium homeostasis dysregulation, eventually resulting in neuronal damage and death. Thus, agents that can stabilize mitochondrial structure and function are considered to have the capability of stopping PD progression (Denzer et al., 2015; Moon and Paek, 2015). However, since mitochondrial protein and/or DNA structure, for the most part, are irreversibly damaged/alteration in PD progression (Brauer et al., 2015; Mäkelä et al., 2016), the agents always provide only limited neuronal protection.

Supplement of functional mitochondria is the most direct approach to replace the damaged mitochondria of PD. It has been established that isolated mitochondria can enter cultured mammalian cells (Clark and Shay, 1982), and rescue respiration in human A549 lung carcinoma cells devoid of mtDNA (Katrangi et al., 2007). Further study also indicates that normally myocardial mitochondria injected into the ischemic zone of myocardial tissue during early reperfusion significantly can enhance regional and global postischemic functional recovery (McCully et al., 2009). Therefore, we are assuming that the PD progress should be prevented, provided that the damaged mitochondria of PD were replaced by systemically administration of functional exogenous mitochondria.

In this study, we first identify that isolated mitochondria from human hepatoma cell have the ability to enter neuronal cells, and determine that the mitochondria can be systemically administrated into

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animals. Then we examine the effects of exogenous mitochondria on animal behaviors, and reach the conclusion that mitochondrial therapy would be a promising strategy for PD treatment. It's the first time to suggest that systemic administration of mitochondria could be used for mitochondrial diseases.

2. Materials and methods

2.1. Animals

Healthy male mice, C57BL/6J species (SCXK [Jing 2006–2009]), weighing 25–30 g, were used in the study. Healthy male null mice (SCXK [Jing 2009–0015]), weighing 25–30 g, were used for *In Vivo* Imaging. Both species were purchased from Chongqing Medical University, Chongqing, China. Animals were maintained under standard housing conditions with *ad libitum* access to standard laboratory mouse chow and water. All animal experiments were carried out in accordance with guidelines evaluated and approved by the Animal Committee of Southwest University, China.

2.2. Isolation of mitochondria

HepG2 cells were cultured according to the experimental protocol (Wu et al., 2015). The plasmid pCMV/mito/GFP (Invitrogen, Cambridge, MA), encoding a fusion protein of GFP and mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase, was transduced into the HepG2 cells by using transfection reagent Lipofectamine 3000 (Invitrogen, Cambridge, MA). The cells were stably transfected with the plasmid according to manufacturer's manual, and the stability was preserved by selection with 400 μg/mL G418 (Gibco, USA).

The HepG2 cells were digested by 0.25% trypsin/EDTA at 37 °C for 5 min, then washed by PBS for twice. Mitochondria isolation was performed according to the manufacturer's protocol of mitochondrial isolation and purification kit (Pierce, Wisconsin, USA). Briefly, the cells was homogenized in cold Isolation Buffer, and then the homogenate was centrifuged at 800*g* for 5 min to move unbroken cells and cell debris. Subsequently, the supernatant was collected and resuspended in Isolation Buffer for another centrifugation at 10000*g* for 10 min. The mitochondria precipitate was washed with Isolation Buffer for 2 times and placed at 4 °C before use. Mitochondrial concentration was determined by BCA assay.

2.3. Characteristics of the mitochondria

The mitochondria isolated from HepG2 cells (without plasmid transfection) was stained with mitochondrial specific indicator, Mitotracker red CMXRos (0.1 μmol/L; Invitrogen, Cambridge, MA) according to the manufacturer's protocol, since Mitotracker red CMXRos can form covalent adducts with protein thiols of mitochondria. Fluorescent mitochondria were spotted onto slides and photographed using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The mitochondrial membrane potential $\Delta\Psi_m$ was detected by JC-1 assay kit (Jiangsu kaiji Biotech. Ltd. Co, Nanjing, China), and florescence intensity was recorded on a fluorescence spectroscopy (HORIBA Scientific USA).

Mitochondria swelling was determined by Ca^{2+}-induced swelling according to previous report (Gunter and Pfeiffer, 1990) in order to identify whether the mitochondria remained intact after isolation. Fresh prepared mitochondria (0.5 mg/mL) is added into the homoiosmotic buffer (140 mmol/L KCl, 10 mmol/L Tris-Cl, pH 7.4) for 60 min incubation, then 10 mmol/L Pi(K), 10 mmol/L succinic acid and 6.5 mmol/L $CaCl_2$ were added in room temperature to initiate mitochondrial swelling. The absorbance (520 nm) were respectively recorded at 1, 2, 4, 6, 8, 10 min following $CaCl_2$ addition.

2.4. Stability of mitochondria in serum

Blood samples were collected from healthy mice into Vacutainer tubes. For serum preparation, the samples was allowed to sit for an hour to clot, then serum was separated. The serum was respectively divided in 400 μL aliquots into sterile Eppendorf tubes. Mitochondria were added into the serum and incubation at 37 °C for 0, 15, 30, 60, 120 min, respectively. Afterwards, samples were centrifuged at 3000*g* for 10 min at 4 °C. The mitochondria were collected and measured by Mitotracker red CMXRos or GFP, and membrane potential assay.

2.5. Cell uptake

Human neuroblastoma SH-SY5Y cells were cultured according to the previous reports (Zhang and Fu, 2015). In brief, these cells were maintained as monolayers in Dulbecco's modified Eagle medium (DMEM) or DMEM/F-12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL Streptomycin. All media were obtained from Gibco. The cells were cultured in an incubator (ESCO, Indonesia) at 37 °C under a humidified atmosphere containing 5% CO_2.

Cells were cultured in a 24-well plate at a density of 5×10^4 cells/well overnight, and then the isolated mitochondria labeled by Mitotracker red CMXRos or GFP were added into the cell media (containing 10% FCS). Following 2 h incubation, the cell media were washed 3 times and replaced by fresh DMEM. The cells were observed and photographed using a confocal microscope (Zeiss LSM 510, Zeiss, Germany).

2.6. Cell viability and biochemical assay

SH-SY5Y cells were cultured at a density of 5×10^4 cells/well in 96-well plates. When cell confluence reached 85%, different concentrations of mitochondria were added into the cell media. Then cell media were replaced with fresh DMEM, and cell viability was measured using AlamarBlue® Cell Viability Assay Reagent (Pierce, USA), according to the manufacturer's protocol. The absorbance was recorded at 630 nm on the microplate reader. Cells not being treated with mitochondria were used as control. The relative cell viability was calculated as cell viability (%) = OD_{630} (sample-blank) / OD_{630} (control-blank).

Meanwhile, cellular ATP content and reactive oxygen species (ROS) level were respectively measured using commercial kits (Nanjing Jiancheng Biotech. Ltd. Co., Nanjing, China). The values were averaged from four independent experiments.

2.7. MPP^+-induced cell injury and mitochondria treatment

When SH-SY5Y cells grew to 85% confluence, 200 μM 1-methyl-4-phenyl-pyridinium (MPP^+) was added into the cell media for 24 h incubation according to previous reports (Lee et al., 2012). After the media were replaced by fresh DMEM, different concentrations of mitochondria were added into the media for another 24 h. Then the cell viability was measured by using AlamarBlue® cell viability reagent (Pierce, USA). Meanwhile, levels of ATP, ROS, GSH and mitochondrial respiratory chain complex I activity were respectively detected as described using commercial kits (Nanjing Jiancheng Biotech. Ltd. Co., Nanjing, China). In addition, cell apoptosis and necrosis were measured by flow cytometry (BD FACSVantage, USA). Four independent experiments were performed for each assay.

2.8. In vivo distribution analysis

The saline solution of fluorescent mitochondria were respectively injected slowly *via* tail veins of nude mice at a dose of 0.5 mg/kg body weight. The mice were euthanized with 4% chloral hydrate following 2 h injection. Afterwards, the mice were transcardically perfused with