Mitochondrial transfer from bone-marrow-derived mesenchymal stromal cells to chondrocytes protects against cartilage degenerative mitochondrial dysfunction in rats chondrocytes

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Abstract

Background: Previous studies have reported that mitochondrial dysfunction participates in the pathological process of osteoarthritis (OA). However, studies that improve mitochondrial function are rare in OA. Mitochondrial transfer from mesenchymal stem cells (MSCs) to OA chondrocytes might be a cell-based therapy for the improvement of mitochondrial function to prevent cartilage degeneration. This study aimed to determine whether MSCs can donate mitochondria and protect the mitochondrial function and therefore reduce cartilage degeneration.

Methods: Bone-marrow-derived mesenchymal stromal cells (BM-MSCs) were harvested from the marrow cavities of femurs and tibia in young rats. OA chondrocytes were gathered from the femoral and tibial plateau in old OA model rats. BM-MSCs and OA chondrocytes were co-cultured and mitochondrial transfer from BM-MSCs to chondrocytes was identified. Chondrocytes with mitochondria transferred from BM-MSCs were selected by fluorescence-activated cell sorting. Mitochondrial function of these cells, including mitochondrial membrane potential (Δψm), the activity of mitochondrial respiratory chain (MRC) enzymes, and adenosine triphosphate (ATP) content were quantified and compared to OA chondrocytes without mitochondrial transfer. Chondrocytes proliferation, apoptosis, and secretion ability were also analyzed between the two groups.

Results: Mitochondrial transfer was found from BM-MSCs to OA chondrocytes. Chondrocytes with mitochondrial from MSCs (MSCs + OA group) showed increased mitochondrial membrane potential compared with OA chondrocytes without mitochondrial transfer (OA group) (1.79 ± 0.19 vs. 0.71 ± 0.12, t = 10.42, P < 0.0001). The activity of MRC enzymes, including MRC complex I, II, III, and citrate synthase was also improved (P < 0.05). The content of ATP in MSCs + OA group was significantly higher than that in OA group (161.90 ± 13.49 vs. 87.62 ± 11.07 nmol/mg, t = 8.515, P < 0.0001). Meanwhile, we observed decreased cell apoptosis (7.09% ± 0.68% vs. 15.89% ± 1.30%, t = 13.39, P < 0.0001) and increased relative secretion of type II collagen (2.01 ± 0.14 vs. 1.06 ± 0.11, t = 9.141, P = 0.0008) and proteoglycan protein (2.08 ± 0.20 vs. 0.97 ± 0.12, t = 8.227, P = 0.0012) in MSCs + OA group, contrasted with OA group.

Conclusions: Mitochondrial transfer from BM-MSCs provided protection for OA chondrocytes against mitochondrial dysfunction and degeneration through improving mitochondrial function, cell proliferation, and inhibiting apoptosis in chondrocytes. This finding may offer a new therapeutic direction for OA.

Keywords: Mitochondrial transfer; Osteoarthritis; Mitochondrial dysfunction; Chondrocyte; BM-MSCs

Introduction

Osteoarthritis (OA) is a common aging-associated degenerative joint disease attributed to degeneration of articular cartilage, joint pain, and functional impairment.1-3 As the sole functional cells present in mature cartilage, the chondrocytes regulate plenty of physiological processes, including the synthesis, deposition, and modification of the extracellular matrix.4-5 However, the molecular mecha-
decreased MRC activity and reduced mitochondrial membrane potential ($\Delta \psi _m$) in OA chondrocytes.[14]

Mitochondrial dysfunction affects several pathways involved in OA pathology, including chondrocyte apoptosis, oxidative stress, cytokine-induced chondrocyte inflammation, and calcification of the cartilage matrix.[2,6,7] Therefore, enhancing mitochondrial activity may be a therapeutic alternative for OA patients. Mitochondrial transfer from donor cells into injured cells has been proposed as a cell-based therapy for the improvement of mitochondrial activity.[8-10] However, studies that regulate mitochondrial function are not currently mentioned in OA.

Therefore, our study investigated whether mitochondrial transfer from mesenchymal stem cells (MSCs) to OA chondrocytes exist. And whether the mitochondrial transfer could improve the mitochondrial function of OA chondrocytes and attenuate cartilage degeneration.

**Methods**

**Ethical approval**

This study was approved by the Institutional Ethical Committee for Animal Care and Use of Peking University First Hospital (No. 201866). All animals were provided by the Laboratory Animal Centre of Peking University First Hospital, and this study was also performed in the Laboratory Animal Centre of Peking University First Hospital.

**Bone-marrow-derived mesenchymal stromal cells (BM-MSCs)**

According to the protocol previously described, BM-MSCs were harvested from 2-week old male rats and cultured.[11] In brief, rats femurs and tibia were cut off and the marrow cavities were flushed using sterile phosphate buffer saline in aseptic condition. The harvested cell suspension was filtered and isolated. BM-MSCs were cultured with Dulbecco modified Eagle medium (DMEM) (HyClone, Ge Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, GE Healthcare Life Sciences) in a 37°C incubator with 5% CO₂ atmosphere, 3rd to 5th passage cells were used in subsequent studies.

**OA chondrocytes**

OA animal model was established according to the protocol previously described.[12] Briefly, 8-week old rats were anesthetized by pentobarbital (5 mg/kg, intraperitoneal injection), bilateral anterior cruciate ligament and medial meniscus were resected to induce the pathogenesis of OA. The rats were sacrificed eight weeks later. Cartilage samples were gathered from the femoral and tibial plateau, and chondrocytes were obtained using the modified Klagsbrun method. After cut into small pieces (1 mm³), the cartilage was incubated with 0.4% Hank balanced salt solution (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 2 h. Further digestion was done with sterile 0.2% collagenase (Nanjing KeyGen Biotech Co., Ltd.) for another 5 h. Then, cell suspension was filtered two times, and isolated chondrocytes were cultured in DMEM (Hyclone), mixed with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Nanjing KeyGenBiotech Co., Ltd.) for 24 h. The cells were incubated in a humidified atmosphere at 37°C, containing 5% CO₂. For cell synchronization, chondrocytes were cultured by serum starvation for 24 h.

**Co-culture of OA chondrocytes and BM-MSCs**

Mitochondrial targeting green fluorescence protein (lentiviral-mediated mitochondrial-specific fragment fused with green fluorescence protein, LV-Mito-GFP, Cat. No. Cyto102-PA-1, System Biosciences, Palo Alto, CA, USA) was transfected. Briefly, 5 x 10⁵ BM-MSCs were seeded before infection, 24 h later, 1 mL growth medium with LV-Mito-GFP was added to cells and incubated for 16 h. LV-Mito-GFP vector, encoding a mitochondrial cytochrome oxidase subunit VIII and a fusion of GFP, will allow us to distinguish mitochondrial trafficking. Chondrocytes were labeled with CellTrace Violet. Labeled OA chondrocytes and labeled MSCs were co-cultured for 24 h at a ratio of 1:1. The co-cultured cells were harvested and the chondrocytes merged labeled mitochondrial targeting green fluorescence protein were selected by flow cytometry (BD FACS Aria III, BD Biosciences, Franklin Lakes, NJ, USA). For contrast, labeled chondrocytes which were not co-cultured with BM-MSCs were also selected.

**Transmission electron microscope (TEM) observation of chondrocytes**

Chondrocytes were prepared as a monoplast suspension of >1 x 10⁵ cells/mL and were fixed by glutaraldehyde for 24 h and then osmic acid for 2 h. The chondrocytes were embedded by resin after dehydration and stained with uranium acetate and lead citrate. The morphology of the chondrocytes, including organelles, the number and shape of mitochondria, the electron density in the outer membrane, and the cristae of the inner membrane was then observed.

**Mitochondrial membrane potential ($\Delta \psi _m$) measurement of chondrocytes**

OA chondrocytes with (MSCs + OA group) or without (OA group) merged labeled mitochondrial targeting green fluorescence protein were harvested. After preparing a monoplast suspension with density more than 1 x 10⁵ cells/mL, cells were stained with 5,5’,6,6’-tetrachloro-1,1’,3’-tetraethyl-imidacarbocyanine (JC-1) at 37°C for 20 min, according to the operation instruction of the $\Delta \psi _m$ assay kit (Sigma-Aldrich, St Louis Missouri, USA). JC-1 is a carbocyclic lipophilic fluorescent dye which is sensitive to membrane potential, it can form polymers in the mitochondrial matrix of normal membrane potential cells and exhibit bright red fluorescence emission. In apoptotic cells, however, the membrane potential decreased, JC-1 forms as monomer and exhibits green fluorescence emission. Flow cytometry was performed to measure the
$ \Delta \psi_m$ within 30 min after staining. The JC-1 monomer was detected under excitation wavelength at 488 nm and emission wavelength at 530 nm, and the JC-1 polymer was observed using excitation wavelength at 490 nm and emission wavelength at 590 nm. Data were analyzed using FlowJo (version 7.6.5, BD Biosciences).

**Identification of respiratory chain complex activity and ATP content**

In order to investigate if the mitochondrial transfer from BM-MSCs could improve the mitochondrial function of OA chondrocytes, quantitative analysis of the MRC enzymes’ activities, including MRC complexes I, II, III, and citrate synthase were determined. Quantification of the ATP was also detected. OA chondrocytes of two groups were prepared as a monoplast suspension with a density of more than $1 \times 10^7$ cells/mL. According to the protocol of mitochondrial extraction kit (Nanjing KeyGen Biotech Co., Ltd.), mitochondria were extracted. The activities of the mitochondrial enzymes and the content of ATP were calculated using the initial velocity equation for pseudo-first-order reactions at 25°C.

**Measurement of cell proliferation and apoptosis**

Cellular proliferation of chondrocytes was assessed with Cell Counting Kit-8 (Applygen Technologies, Inc., Beijing, China). Optical density values of the cells between the two groups were measured and compared on days 1, 3, 5, and 7. For apoptosis, chondrocytes were stained by propidium iodide and Annexin V-enhanced green fluorescent protein at 37°C for 15 min, then cells were assessed by flow cytometry (BD Biosciences) with Annexin V-fluorescein isothiocyanate kit (Nanjing KeyGen Biotech Co., Ltd.). Data collected were then analyzed with FlowJo (version 7.6.5, BD Biosciences).

**Quantitative analysis of type II collagen and proteoglycan protein**

Total protein in chondrocytes was extracted by the total protein extraction kit (Nanjing KeyGen Biotech Co., Ltd.). The quantification of type II collagen and proteoglycan was determined via Western blotting. Briefly, the concentration was measured with Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) by running on 10% polyacrylamide gel. Polyvinylidene fluoride membranes with protein were blocked and incubated with the primary antibodies, collagen II (ab34712, Abcam, Cambridge, UK), proteoglycan (ab2501, Abcam), and glyceraldehyde-3-phosphate dehydrogenase (ab128915, Abcam), overnight at 4°C. Then, rabbit anti-mouse secondary antibody was incubated at 37°C for 1 h.

**Statistical analysis**

Statistical analysis was operated with SPSS software (version 23.0; IBM Corp., New York, USA). All data were reported as the mean ± standard deviation. Student’s $t$-test with subsequent Bonferroni correction was adapted to compare results from two groups, with $P < 0.05$ considered statistically significant.

### Results

**Mitochondrial transfer from BM-MSCs to OA chondrocytes**

MSCs with LV-Mito-GFP were successfully built to reveal the mitochondrial transfer [Figure 1A]. OA chondrocytes were labeled with CellTrace Violet [Figure 1B]. OA chondrocytes (violet) and BM-MSCs (green) can be distinguished. After 24 h co-culture of these two kinds of cells, Mito-GFP was observed in many violet fluorescence-positive cells [Figure 1C], indicating that mitochondria from MSCs had translocated to chondrocytes.

**TEM observation of chondrocytes**

Chondrocytes of MSCs + OA group were selected by flow cytometry [Figure 1D] and chondrocytes not co-cultured (OA group) were selected for observation by TEM. In MSCs + OA group, various organelles were visible, including the endoplasmic reticulum, the Golgi apparatus, and the number of mitochondria was much more than OA chondrocytes. The shape of mitochondria was oblong. In the outer membrane, the electron density was continuous and high, and in the inner membrane, the cristae was regularly arranged [Figure 2A]. However, in OA group, the mitochondria were more swollen, and its shape was more round rather than oblong. In the outer membrane, the electron density became discontinuous and asymmetrical, while in the inner membrane, the cristae exhibited an irregular pattern, or even cannot be detected. [Figure 2B].

**$ \Delta \psi_m$ measurement of chondrocytes**

Compared with OA group, the red fluorescence in MSCs + OA group was increased, while the green fluorescence was decreased [Figure 2C and 2D]. The ratio of red/green fluorescent signal in MSCs + OA group was 1.79 ± 0.19, whereas the signal in OA group was 0.71 ± 0.12. Importantly, the differences between MSCs + OA and OA groups showed statistically significant ($t$ = 10.42, $P < 0.01$) [Figure 2E].

**Detection of respiratory chain complex activity and ATP content**

The activities of the MRC complexes I, II, III, and citrate synthase in MSCs + OA group were remarkably increased compared with OA group ($69.11 \pm 16.69$ vs. $30.61 \pm 6.24$ nmol-min$^{-1} \cdot$mg$^{-1}$, $t = 3.174$, $P = 0.013$; $36.67 \pm 5.65$ vs. $12.52 \pm 2.96$ nmol-min$^{-1} \cdot$mg$^{-1}$, $t = 6.323$, $P = 0.0002$; $118.74 \pm 9.50$ vs. $47.61 \pm 8.94$ nmol-min$^{-1} \cdot$mg$^{-1}$, $t = 6.523$, $P = 0.0002$; and $196.40 \pm 26.18$ vs. $115.00 \pm 15.37$ nmol-min$^{-1} \cdot$mg$^{-1}$, $t = 5.362$, $P = 0.0007$, respectively).

The content of ATP in OA group was $87.62 \pm 8.94$ nmol/mg. Whereas in MSCs + OA group, it was $161.90 \pm 13.49$ nmol/mg, which was significantly increased compared with OA group ($t = 8.515$, $P < 0.01$). The present results proposed that mitochondrial transfer from BM-MSCs was able to improve mitochondrial function of OA chondrocytes.
Cellular viability assay

Cell proliferation assay revealed that the proliferative ability of MSCs + OA group was significantly increased compared with OA group on days 3, 5, 7 (all \( P < 0.05 \)) [Figure 3A]. Meanwhile, the total apoptosis rate in OA group was 15.89% ± 1.30% (The early and late apoptosis rates were 1.54% ± 0.91% and 14.35% ± 1.25%, respectively) [Figure 3B]. In MSCs + OA group, the early, late, and total apoptosis rates were 1.85% ± 0.71%, 5.24% ± 0.99%, and 7.09% ± 0.68%, respectively [Figure 3C]. Significant difference in total apoptosis rates can be detected between two groups (\( t = 13.39, P < 0.01 \) [Figure 3D]). These results suggested that improved mitochondrial function was able to enhance cell proliferation and decrease the apoptosis rate of OA chondrocytes.

Quantitative detection of type II collagen and proteoglycan protein

Type II collagen and proteoglycan protein levels were significantly elevated in MSCs + OA group than those in OA group determined (2.01 ± 0.14 vs. 1.06 ± 0.11, \( t = 9.141, P = 0.0008 \); and 2.08 ± 0.20 vs. 0.97 ± 0.12, \( t = 8.227, P = 0.0012 \), respectively) [Figure 4].

Discussion

As a progressive joint degenerative disease, OA may often lead to physical disability. Mitochondrial dysfunction may influence the pathogenesis of OA and the function of chondrocytes. Cillero-Pastor et al.\(^{[13]}\) reported that inhibiting complex III activity using antimycin-A (a specific MRC inhibitor) could increase the apoptosis in human chondrocytes. Several studies have identified that specific MRC inhibitors suppressed the secretion of collagen and proteoglycans in human chondrocytes.\(^{[14-16]}\) So, improving mitochondrial function may be a therapeutic alternative for OA. MSCs have been recommended as a valuable cell source in the therapy of OA, which exert therapeutic functions mainly via anti-inflammatory actions, paracrine, and cartilage repair.\(^{[17-19]}\) Demonstrated as an important mechanism of MSCs therapy, the mitochondrial transfer is associated...
with the rescue of aerobic respiration and restoration of mitochondria function in the recipient cells.\cite{8,9,20,21} It played a critical role during disease healing processes, such as cerebral injury, cardiac myopathies, lung diseases, and acute respiratory disorders.\cite{20-24} However, the potential mechanism of mitochondrial function improvement in OA through MSCs therapy is not currently included. In our study, we show for the first time that mitochondrial transfer can occur from BM-MSCs to OA chondrocytes \textit{in vitro}. These results also revealed that functional mitochondrial transfer from MSCs can directly protect chondrocytes against mitochondrial dysfunction and reverses states of OA chondrocytes, which is a critical mechanism in MSC therapy.

TEM imaging provides the visualization of mitochondrial morphological features. The morphology and number of the cristae in the mitochondrial inner membrane are associated with cell metabolism. Ma \textit{et al}.\cite{25} revealed that the loss of normal mitochondrial morphology may be associated with the dysfunction of the MRC, including the uncovering of the mitochondrial permeability transition pore, the decrease of $\Delta \psi m$ and the collapse of the outer membrane. In our study, we found more normal mitochondria in MSCs + OA groups compared with OA chondrocytes. This result suggested that MSCs can transfer healthy mitochondria to OA chondrocytes, which can hence improve the mitochondrial dysfunction of OA chondrocytes.

Figure 2: Transmission electron microscope and $\Delta \psi m$ results. (A) Transmission electron microscope results in MSCs + OA group. Organelles were visible in the cytoplasm, including a large number of mitochondria, the endoplasmic reticulum, and the Golgi apparatus. The shape of mitochondria was oblong. The electron density in the outer membrane was continuous and high, the cristae in the inner membrane was regularly arranged. Scale bar = 0.5 $\mu$m. (B) Transmission electron microscope results in OA group. The number of organelles in the cytoplasm was decreased compared with MSC + OA group. The mitochondria were more swollen, the electron density was discontinuous and the structure of the cristae was disarrayed (arrow). (C and D) Immunofluorescence staining of $\Delta \psi m$ in OA and MSCs + OA groups, respectively. Original magnification, $\times$1000. (E) The ratios of red/green fluorescent signal in OA group and MSCs + OA group were $0.71 \pm 0.12$ and $1.79 \pm 0.19$, respectively ($P < 0.01$, OA group vs. MSCs + OA group). $\Delta \psi m$: Mitochondrial membrane potential; MSCs: Mesenchymal stromal cells; OA: Osteoarthritis.
Mitochondrial dysfunction participates in the process of chondrocyte apoptosis, while numerous preclinical studies indicated that chondrocyte apoptosis can be reversed by mitochondrial modulation. Previous studies proved that mitochondrial transfer could increase ATP content and enhance gene and protein expression relating to the tricarboxylic acid cycle, thus protect against cell injury and apoptosis. Here, we also exhibited that mitochondrial transfer from MSCs to OA chondrocytes increased the activity of the complexes of MRC, mitochondrial membrane potential, and ATP content. Meanwhile, we indicated that mitochondrial transfer was an important strategy in preventing the chondrocytes from mitochondrion-driven apoptosis. In our study, cell proliferation increased by about 98% in the 7th day, while total apoptosis rates decreased by about 35%, secretion level of type II collagen and proteoglycan protein improved about 1-fold in MSCs + OA group. This finding confirmed that mitochondrial transfer from MSCs to OA chondrocytes can significantly increase the mitochondrial function and ameliorate degeneration of OA chondrocytes.

There are several limitations in this study. First, the relative roles of paracrine secretion vs. mitochondrial transfer in the
BM-MSCs protective effect were not mentioned. Jiang et al. indicated that paracrine action and mitochondrial transfer were two independent processes, which collaborated to MSC-mediated protection. Second, the potential mechanisms of mitochondrial transfer from BM-MSCs to chondrocytes in our study were not elucidated. Many studies have indicated that various mechanisms including tunnel tube formation, gap junctions, microvesicle formation, cell fusion, and other modes of transfer are involved in mitochondrial transfer. Further studies are needed in terms of mitochondrial transfer from MSCs to chondrocytes.

In conclusion, our study reveals that mitochondrial transfer from BM-MSCs might protect OA chondrocytes against mitochondrial dysfunction and cartilage degeneration through improving mitochondrial function, cell proliferation, and inhibiting apoptosis in chondrocytes. This finding may offer a new therapeutic direction for OA. Further studies are needed in the mechanism of mitochondrial transfer from MSCs to chondrocytes.

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Conflicts of interest
None.

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