Scientific Research Publishing

Journal of Biomaterials and Nanobiotechnology, 2024, 15, 65-77
https://www.scirp.org/journal/jbnb
ISSN Online: 2158-7043
ISSN Print: 2158-7027

Cryopreserved Fibroblast and Mesenchymal Stem Cells (MSCs) Being Alternative Mitochondrial Donors for Mitochondrial Organelle Transplantation (MOT)

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How to cite this paper: Jiang, X.P., Segal, B., Kindy, M.S. and Baucom, C.C. (2024) Cryopreserved Fibroblast and Mesenchymal Stem Cells (MSCs) Being Alternative Mitochondrial Donors for Mitochondrial Organelle Transplantation (MOT). *Journal of Biomaterials and Nanobiotechnology*, **15**, 65-77.
https://doi.org/10.4236/jbnb.2024.154005

Received: September 6, 2024
Accepted: October 15, 2024
Published: October 18, 2024

Abstract

Mitochondrial organelle transplantation (MOT) is an innovative strategy for the treatment of mitochondrial dysfunction such as cardiac ischemic reperfusion injuries, traumatic brain and spinal cord injuries, cerebral stroke, and neurodegenerative diseases. The earlier MOT results in better efficacy in animal models of urgent diseases such as ischemic stroke, and traumatic brain and spinal cord injuries. There is no long-term method to preserve mitochondria. Routine MOT procedure from cell growth to mitochondrial injection often takes serval weeks and is not satisfactory for urgent use cases. **Hypothesis:** Cryopreserved cells might be mitochondrial donors for MOT. **Methods:** We isolated mitochondria from cryopreserved human fibroblasts and mesenchymal stem cells (MSCs) in cell banks and compared the mitochondrial viability and transplantation with the mitochondria from fresh cells. **Key findings:** We found that mitochondria from fresh and cryopreserved cells are comparable in mitochondrial viability and transplantation. We also obtained data showing that mitochondria of fibroblasts and MSCs had similar membrane potential and transfer ability, but MSC's mitochondria had higher ATP content than fibroblast's mitochondria. In addition, oxygen consumption rates (OCRs) were higher in MSC's mitochondria compared to fibroblast's mitochondria and did not change between fresh and frozen cells. **Conclusion:** Cryopreserved fibroblasts and MSCs are alternative mitochondrial donors for MOT to fresh cells. MSCs could provide higher ATP-produced mitochondria than fibroblasts.

Keywords

Mitochondria, Mitochondrial Organelle Transplantation, MOT, Cryopreservation, Fibroblasts, MSCs

1. Introduction

Mitochondria are powerhouses to produce ATP by oxidative phosphorylation (OXPHOS) in the presence of oxygen. They are also associated with the synthesis of iron-sulfur clusters and heme, β-oxidation of fatty acids, and homeostasis of calcium, iron and reactive oxygen species (ROS) [1]-[3]. Mitochondrial dysfunction plays an important role in many diseases such as cardiovascular disease, metabolic disease, neurodegenerative disease, ischemic reperfusion injuries, and traumatic brain and spinal cord injuries, etc. [3]-[6].

In recent years, mitochondrial organelle transplantation (MOT) has been reported as an innovative therapeutic intervention that benefits neuronal survival and regeneration for neurodegenerative diseases, ischemic reperfusion injuries, and traumatic brain and spinal cord injuries [7]-[15]. Masuzawa *et al.* have extensively studied the role of injected isolated mitochondria for cardio protection during ischemia-reperfusion. The exogenous mitochondria could enter into cardiomyocytes within 2 hours after injection and maintain viability and function producing adequate ATP levels. They also demonstrated that the exogenous mitochondria provided cardio protection both extracellularly and intracellularly [8]. Huang and colleagues demonstrated that local intracerebral or systemic intra-arterial injection of isolated hamster mitochondria into brain-ischemic rats significantly reduced neuronal death and restored motor performance. They found that the mitochondrial internalization to neurons could not completely account for the high rescue of neuronal injury. Extracellularly exogenous mitochondria may be a source of ATP and a ROS scavenger to protect cells from damage by free radicals [10]. Transplantation of placenta-derived mitochondria via intravenous infusion significantly decreased brain infarction after focal cerebral ischemia in mice [11]. In animal experiments of traumatic brain and spinal cord injury, transplantation of allogeneic mitochondria at the early stage of spinal cord injury (SCI) reduces mitochondrial fragmentation, neuro-apoptosis, neuroinflammation, and generation of oxidative stress, thus leading to improved functional recovery following traumatic SCI [12] [13]. Mitochondria transplantation also significantly reduced neuronal death and memory impairment following traumatic brain injury (TBI) [14] [15]. All above mitochondrial transplantation were performed shortly after ischemic injury, and traumatic brain or spinal cord injury. The earlier MOT results in better efficacy in animal models of these urgent diseases. Routine MOT procedure includes cell expansion for several weeks, mitochondrial isolation and subsequent patient injection. The routine MOT is not ideal for urgent usage in ischemic-reperfusion stroke, and traumatic brain or/and spinal cord injuries.

Human cells can be kept in liquid nitrogen for long-term storage. If cryopreserved cells provide viable intact mitochondria, MOT could be adequate for urgent treatment of diseases. To prove the hypothesis, we isolated mitochondria from cryopreserved human fibroblasts and mesenchymal stem cells (MSCs) in cell banks and compared the mitochondrial viability and transfer ability with the mitochondria from fresh cells. We found that mitochondria from cryopreserved cells are similar to the mitochondria from fresh cells in viability and transplantation. We also obtained results showing mitochondria of fibroblasts and MSCs had similar membrane potential and transfer ability, but MSC's mitochondria had more ATP content than fibroblast's mitochondria.

2. Materials and Methods

2.1. Fibroblast, Mesenchymal Stromal Cell (MSC) and NSC34 Cell Expansion

Human primary fibroblasts were established and stored in liquid nitrogen [16]. Human primary fibroblasts were recovered from liquid nitrogen and cultured in alpha MEM (GIBCO, Carlsbad, CA, USA) containing 5% human platelet lysate (HPL) (Mill Creek Life Sciences, Rochester, MN, USA). Human MSCs were derived from bone morrow and obtained from RoosterBio (Frederick, MD, USA). MSCs were recovered from liquid nitrogen and cultured in the complete Rooster media (Frederick, MD, USA). NSC-34 is a hybrid cell line, produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma. NSC-34 was purchased from Cedarlane Corporation (Ontario, Canada) and cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) [16]. When cells grew to 80% full in flask, they were digested with TrypLE expression solution (GIBCO, Carlsbad, CA, USA) and sub-cultured at 37˚C and 5% CO_2.

2.2. Mitochondrial Staining with JC-1

Mitochondrial membrane potential (MMP) generated by proton pumps is an essential component in the process of energy storage during OXPHOS. Membrane potential dependent dyes such as JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide) and MitoTracker dyes have been used to stain mitochondria and monitor mitochondrial potential. For JC-1 staining, mitochondria were stained with mitochondria staining kit (Sigma CS0390, St. Louis, MO, USA). The protocol was referred to the document of the product's manufacturer. Fibroblasts and MSCs were incubated with JC-1 solution for 20 minutes at 37˚C in humidified atmosphere containing 5% CO_2. Fluorescence was observed by Olympus IX83 fluorescent microscope. In cells which maintained electrochemical potential gradient, the dye concentrates in mitochondria, where it formed bright red fluorescent aggregates (J-aggregates). If cells failed to maintain MMP, the JC-1 was dispersed through the entire cells resulting in a shift from red to green fluorescence (JC-1 monomers). Cells were treated with mitochondrial inhibitors,

valinomycin or FCCP. The treated cells were controls of mitochondrial dissipation.

2.3. Isolation of Mitochondria

Mitochondrial isolation followed a previously described protocol [16]. All reagents were sterile. 50×10^6 Fibroblasts or MSCs were centrifuged for 5 minutes at 400 g and at 4˚C to remove the media. Cell pellet was re-suspended in ice-cold 300 mM sucrose mitochondrial isolation buffer (MIB) (Sigma Aldrich, St. Louis, MO, USA) and homogenized by bead beating (Bead Ruptor 12, Omni International homogenizer company, Kennesaw, GA, USA). The cell lysate was centrifuged for 10 minutes at 700 g and at 4˚C. Then the supernatant was transferred to new centrifugation tubes and centrifuged for 10 minutes at 9000 g at 4˚C. The supernatant was removed. The wet weight of mitochondria was measured [16]. The mitochondrial pellet was re-suspended with 240 mM sucrose mitochondrial respiration buffer (MRB) (Sigma Aldrich, St. Louis, MO, USA). Mitochondrial suspension was cooled in wet ice. MMP and ATP content of the mitochondria were stable for 24 hours. MMP and mitochondrial ATP content were measured within 2 hours after isolation in the current study.

2.4. Measurement of MMP

The stock solution of JC-1 was added to mitochondrial suspension to a final concentration 1 μg/ml. The mixture was incubated for 10 minutes at room temperature. Isolated mitochondria were treated with mitochondrial inhibitor valinomycin or FCCP. The treated mitochondria were controls of mitochondrial dissipation. Red fluorescent J-aggregates in intact mitochondria could be observed under fluorescent microscope. The relative fluorescence units (RFU) could be read in multiple plate fluorimeter using end-point method with the setting of Ex/Em: 490 nm/590 nm.

2.5. Measurement of ATP Content in Cells and Isolated Mitochondria

ATP content was measured with ATPlite kit (Perkin Elmer Inc., Waltham, MA, USA). The detailed procedure was referred to the product manual. The brief method was as follows: added 50 μl of mammalian cell lysis solution to 100 μl of fibroblasts or MSCs, mitochondria, or MRB per well in a 96-well plate with white well and clear bottom; shook the plate for 5 minutes; added 50 μl substrate solution to all wells and shook the plate for 5 minutes; measured the luminescence of the plate; calculated ATP content of samples using the ATP standard curve.

2.6. Oxygen Consumption Rate (OCR) in Isolated Mitochondria

The respiration assay buffer (mitochondrial assay solution; MAS) contains 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, and 1 mM EGTA. For carrying out respiration using isolated mitochondria, pyruvate

(10 mM) and malate (5 mM) were added to the MAS, and the resulting solution used to make 10× stocks of the respiratory inhibitors and uncouplers. The respiratory stocks were loaded into the drug ports of a hydrated sensor cartridge in the following order: (A) oligomycin (2.5 μg/mL final), (B) FCCP (4 μM final), and (C) antimycin A (4 μM final) + rotenone (2 μM final). The protein concentrations of isolated mitochondria preparations were measured. Equal amounts of mitochondria (1 μg protein/well) were plated on the Seahorse cell culture microplate in 20 μL of MAS + substrate + 0.2% w/v fatty-acid free BSA and centrifuged at 2000×g for 20 min at 4˚C. The assay medium (MAS + substrates + 0.2% BSA + 4.5 mM ADP) was then added to the wells to bring the final volume to 180 μL prior to the plate being incubated at 37˚C for 30 min and transferred to the analyzer for analysis. The respiration assay protocol consisted of a minimum of three cycles of OCR measurements for each measurement period. Each cycle consisted of a 2 min "mix" period and 2 min "wait" period, followed by a 3 min "measure" period. Three cycles were used to obtain a basal OCR, 6 cycles were used to assess the effect of the F1-Fo ATP synthase inhibitor oligomycin, three cycles were used to evaluate the effect of the uncoupler FCCP, and 3 cycles were used to measure mitochondria-associated respiration following injection of antimycin A/rotenone.

2.7. MOT of NSC-34 Cells with MSCs' Mitochondria

Cryogenically frozen MSCs were quickly thawed by gently swirling the vials in the 37˚C water bath. Mitochondria of the recovered MSCs or fresh MSCs were labelled using 150nM of MitoTracker Red dye (Thermofisher Scientific, Waltham, MA, USA) at 37˚C and 5% CO_2 for 30 minutes. The cells were washed with Hanks' balanced salt solution (HBSS) 3 times to remove the dye. Then, mitochondria were isolated. The MitoTracker Red-labelled mitochondria were added to NSC-34 cells that grew on glass surface in glass-bottom culture dishes and incubated overnight at 37˚C and 5% CO_2. Then the NSC-34 cells were washed 3 times with pre-warmed HBSS to remove the labelled mitochondria in the media. The cell dishes were observed under fluorescent microscope.

2.8. Statistical Analysis

Student's t-test was used to test statistical significance by GraphPad Prism. p-value less than 0.05 was judged to be of statistical significance.

3. Results

3.1. Cryopreserved Fibroblasts and MSCs Maintain MMP

Cryovials containing frozen fibroblasts or MSCs were removed from liquid nitrogen storage and immediately placed into a 37˚C water bath. Cells were quickly thawed by gently swirling the vials in the 37˚C water bath until there was just a small bit of ice left in the vials. The vials were transferred to a laminar flow hood. MMP was shown by JC-1 staining. Red fluorescence was observed in mitochondria of the fibroblasts and MSCs. Red fluorescent brightness and distribution in

the cryopreserved cells were not different from the growing fibroblasts and MSCs that attached on surface of slides (Figure 1, Figure 2). Valinomycin or FCCP-treated cells lost most red fluorescence. The data showed that cryogenically frozen fibroblasts and MSCs in liquid nitrogen maintained similar MMP to the fresh fibroblasts and MSCs.

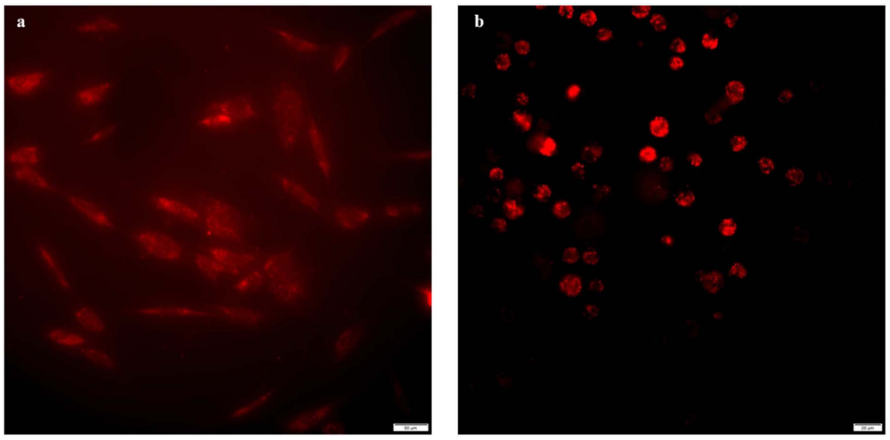

Figure 1. Cryopreserved fibroblasts maintain comparable MMP with fresh fibroblasts. Mitochondria were stained by membrane potential dependent dye JC-1. (a): fresh fibroblasts; (b): cryopreserved fibroblasts.

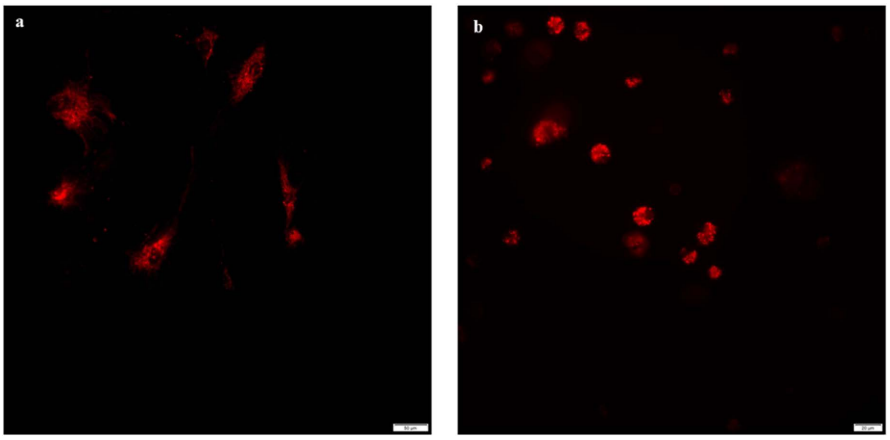

Figure 2. Cryopreserved MSCs maintain similar MMP to fresh MSCs. Mitochondria were stained by membrane potential dependent dye JC-1. (a): fresh MSCs; (b): cryopreserved MSCs.

3.2. Isolated Mitochondria from Cryopreserved Cells Maintain MMP and ATP Content

Vials containing 50×10^6 frozen MSCs in liquid nitrogen or dry ice were immediately placed into a 37˚C water bath by gently swirling the vials until there was just a small bit of ice left in the vials. Mitochondria were isolated from the MSCs and proceeded to measurement of MMP and ATP content. The mitochondria from the cryopreserved MSCs actively took up dye JC-1 and formed bright red fluorescence (J-aggregates) that was similar to the mitochondria isolated fresh MSCs

(Figure 3). In mitochondrial suspension with concentration 100 mg/ml, RFU of the mitochondria of cryopreserved MSCs (435 ± 45) was slightly lower than the mitochondria from fresh MSCs (488 ± 82), but the difference was not significant ($p > 0.05$).

In agreement with MMP, ATP content of the mitochondria from frozen MSCs (51.8 ± 7.7 pmol/mg mitochondria) was slightly less than the mitochondria from fresh MSCs (64.8 ± 8.9 pmol/ml), but the difference was not significant ($p > 0.05$). These results of MMP and ATP content showed that cryopreservation maintained mitochondrial function of MSCs and cryopreserved MSCs were an alternative mitochondrial source to fresh MSCs.

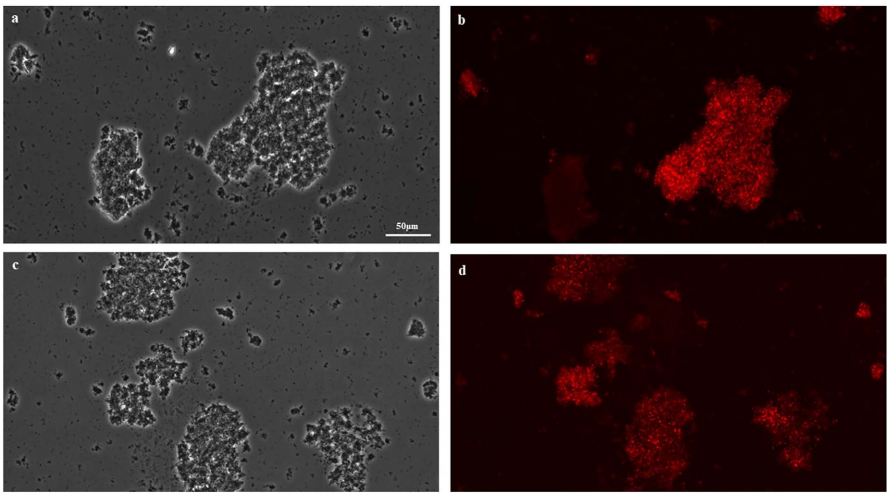

Figure 3. The mitochondria of cryopreserved MSCs maintain comparable MMP to the mitochondria from fresh MSCs. (a), (b): mitochondria of fresh MSCs; (c), (d): mitochondria of cryopreserved MSCs. (a), (c): phase contrast; (b), (d): fluorescent. All images have the same scales.

3.3. Mitochondria Isolated from Cryopreserved MSCs Transfer into NSC34 Cells

We reported previously that isolated mitochondria of human fibroblasts transferred to NSC34 cells after co-culture [16]. After 16 hours of co-culture, Mito-Tracker Red-labelled mitochondria of MSCs were seen in NSC-34 cells under fluorescent microscope. Mitochondria from both fresh and cryopreserved MSCs could enter to NSC-34 cells (Figure 4). The results showed that mitochondria from cryopreserved MSCs had similar ability to transfer into NSC-34 cells to mitochondria of fresh MSCs.

3.4. Fibroblasts and MSCs Being Almost Identical

Morphological characteristics of fibroblasts and MSCs are alike (Figure 5). Both fibroblasts and MSCs have abundant mitochondria indicated by membrane potential dependent dye JC-1 (Figure 1, Figure 2). However, MSCs demonstrated a higher level of ATP than fibroblasts (all were significant at $p < 0.01$, at 1250, 2500,

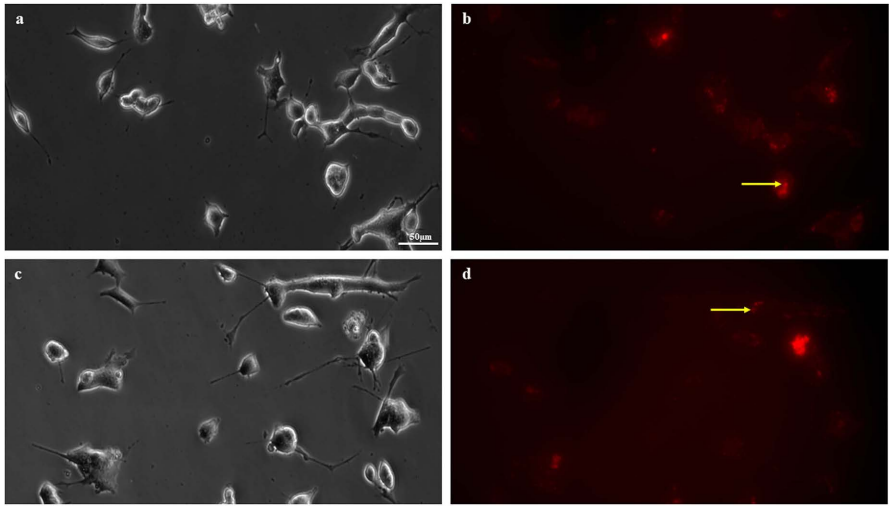

Figure 4. The mitochondria isolated from fresh and frozen MSCs transferred into NSC-34. Mitochondria were labelled with MitoTracker Red dye and isolated from MSCs. The labelled mitochondria were co-cultured with NSC-34 cells overnight. The mitochondria of MSCs were observed by fluorescent microscope. (a), (b): NSC-34 cells co-cultured with the mitochondria of fresh MSCs; (c), (d): NSC-34 cells co-cultured with the mitochondria of cryopreserved MSCs. Arrow: MitoTracker Red labelled mitochondria of MSCs in NSC-34 cells.

5000 and 10,000 cells per wells) (**Figure 6**). We measured MMP and ATP content of mitochondria isolated from fresh fibroblasts and MSCs. We found MMP was not significantly different between the isolated mitochondria of fibroblasts (RFU: 548 ± 108) and MSCs (RFU: 488 ± 82) ($p > 0.05$) (**Figure 7**). In alignment with the ATP content results in whole fibroblasts and MSCs, the isolated mitochondria from MSCs (64.8 ± 8.9 pmol/mg mitochondria) had significantly higher ATP content than the mitochondria of fibroblasts (24.5 ± 1.8 pmol/ml) ($p < 0.01$) (**Figure 8**). Finally, we measured the OCR in the isolated mitochondria from fresh and frozen cells to determine the impact on respiration. Both fresh and frozen fibroblasts (9a) and MSCs (9b) showed similar patterns of OCR and the frozen cells did not show any compromised effect of the cell freezing (**Figure 9**). In addition, as seen in **Figure 6** and **Figure 8**, because of the increased ATP levels in the MSC cells the OCR was higher in the MSC cells as well.

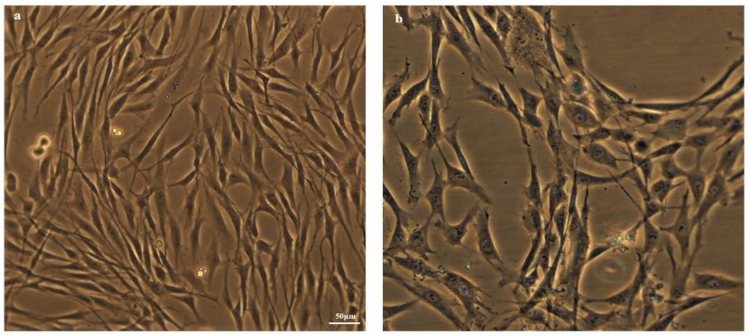

Figure 5. Morphological characteristics of fibroblasts and MSCs are homogeneous. (a): fibroblasts; (b): MSCs.

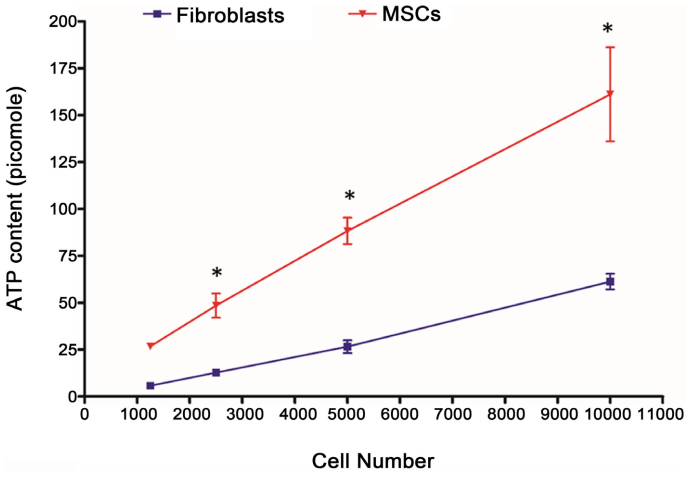

Figure 6. MSCs produce more ATP than fibroblasts. Cells grow on 96-well cell culture plates at 37˚C overnight. ATP content was determined in the different groups. Measurements were repeated 3 times per group, n = 3. The details were seen in the Materials and Methods. *p < 0.01.

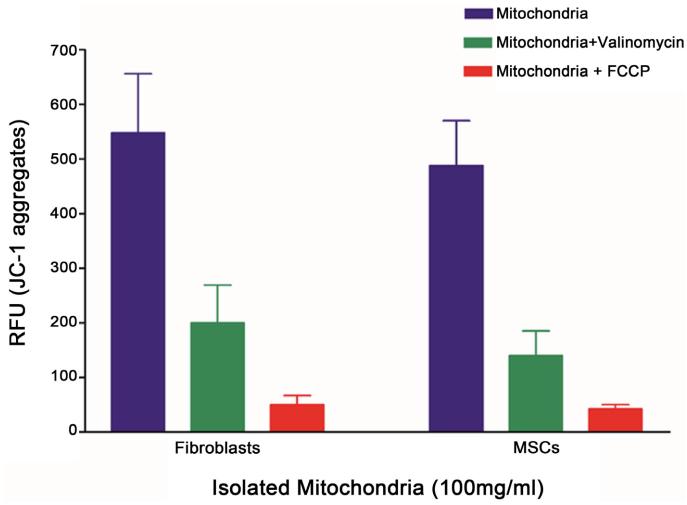

Figure 7. MMP of mitochondria isolated from fibroblasts and MSCs. The relative fluorescence units (RFU) of the mitochondria of fibroblasts and MSCs are not significantly different (p > 0.05). The valinomycin or FCCP-treated mitochondria are the controls of mitochondrial dissipation (Details in the Materials and Methods).

4. Discussion

MOT can replenish mitochondria and mtDNA while restoring mitochondrial function of defective cells [16]. MOT has been shown to yield positive therapeutic results in several disease animal models including rabbit cardiac ischemia-reperfusion [8], rat Parkinson's disease (PD) [9], rat brain ischemia [10], spinal cord injury (SCI) [12], and traumatic brain injury (TBI) [14]. There have been a few MOT clinical trials for the diseases including acute respiratory distress syndrome, myocardial ischemia, ischemia-reperfusion injury and infertility. Benefit of MOT has been observed [17]. However, one of the major challenges for MOT is

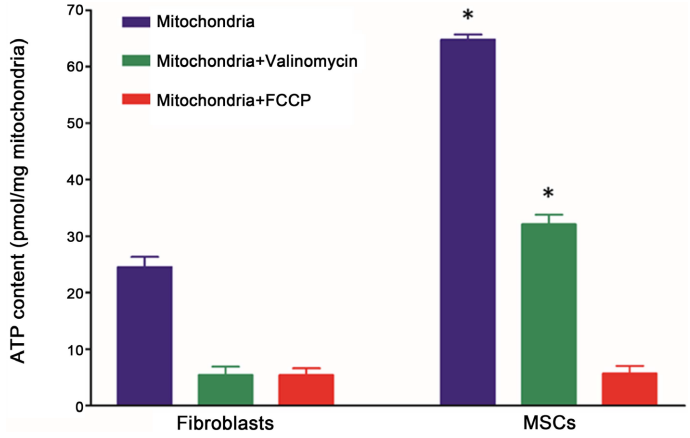

Figure 8. ATP content of mitochondria isolated from fibroblasts and MSCs. The ATP content of the mitochondria from MSCs is significantly higher than the mitochondria of fibroblasts (*p < 0.01). The valinomycin or FCCP-treated mitochondria are the controls of mitochondrial dissipation (Details in the Materials and Methods).

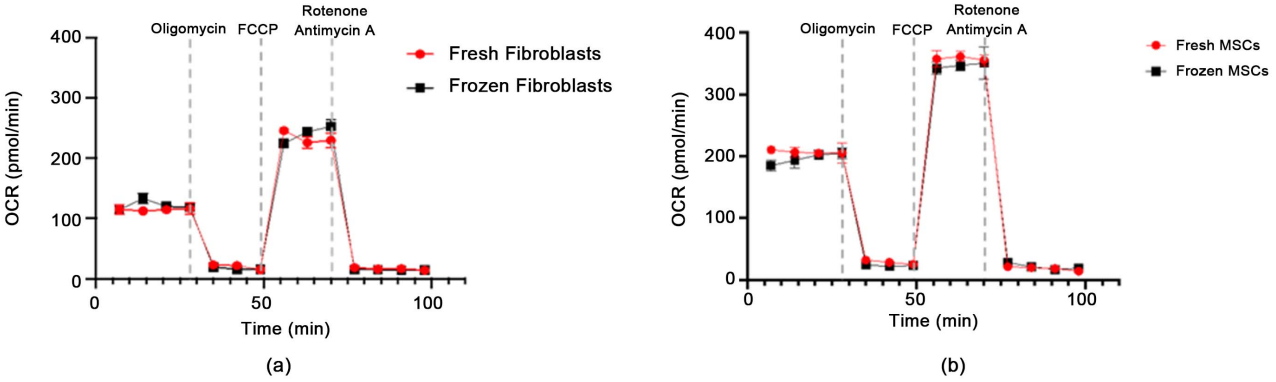

Figure 9. OCR measurements in isolated mitochondria from fresh and frozen fibroblasts and MSCs. Isolated mitochondria from the different cells were subjected to OCR measurements using the Seahorse XF24 analyzer. Fresh and frozen cells were treated as indicated in the Materials and Methods section, and OCR determined. (a) Fibroblasts and (b) MSCs. Assays were repeated 3 times per group.

mitochondrial preservation. The shelf life of isolated mitochondria is short. Cold storage or cryopreservation of mitochondria haven't been successful for long term storage, leading to a decrease in respiratory capacity and damage of mitochondrial membrane structure over time [18]. Therefore, routine MOT procedure includes tissue collection, cell expansion, mitochondrial isolation from fresh cells or tissues, and injection of mitochondria to patients as soon as possible. This method of MOT production has several disadvantages: 1) Requirement of expensive cGMP compliant facility for cell expansion and process in clinical facilities; 2) Cell expansion often takes several weeks. The routine MOT procedure is not practicable for urgent use cases such as ischemic-reperfusion stroke and traumatic brain or/and spinal cord injuries; 3) It is difficult to coordinate the schedule of cell processes and patient need.

The cryogenic banking and shipping of cells with cryoprotectant addition has

removed the need for continuous culture (which results in phenotype drift, as well as consuming large amounts of resources) and enabled successful delivery of emerging cell-based therapy [19]. Even though cryopreservation commonly decreases the cellular functionality including mitochondria and post-thawed viability of cells, cryopreserved cells often maintain high (>90%) viability after thawing. It was reported that the oxygen consumption rate (OCR) measurements of fresh and cryopreserved placental specimens were comparable whereas a snap frozen procedure impairs mitochondrial activity [20]. Kuznetsov AV *et al.* showed intactness of mitochondrial respiratory function after cryopreservation of cardiac and skeletal muscle fibers [21]. In the present study, we found that fresh and cryopreserved fibroblasts and MSCs have comparable MMP, mitochondrial ATP content and OCRs (**Figures 1-3**, **Figure 9**). Moreover, the mitochondria isolated from cryopreserved fibroblasts or MSCs could transfer into NSC-34 neural cells (**Figure 4**). Even though further studies such as mitochondrial ultrastructure remains to be compared in fresh and cryopreserved fibroblasts or MSCs, the current results suggest that cryopreserved cells may be feasible mitochondrial donor to deliver MOT for clinical trials. In order to overcome the disadvantages of routine MOT by using fresh cells for mitochondrial donors, we are exploring an enhanced methodology for MOT clinical trials. Current Good Manufacturing Practice (cGMP)—fibroblasts or MSCs will be expanded and banked in a central cGMP compliant cell factory. The frozen cells will be cryogenically shipped to multiple clinical sites where have installed BioSpherix Xvivo System X2. The system is a portable ISO Class 5 closed aseptic isolator, and designed for producing and processing cells in compliance with regulatory GMPs [22]. Mitochondria will be isolated from the cryopreserved cells in the Xvivo System X2. The isolated mitochondria will be injected to patients after isolation as soon as possible.

We reported a case of MOT study by using human fibroblast's mitochondria. The MOT improved leg muscle strength and recovered all sensory sensation of legs in a patient who suffered from desperate amyotrophic lateral Sclerosis (ALS) [23]. We also noticed that MSCs have been extensively used for cellular therapy. Fibroblasts and MSCs are phenotypically indistinguishable. Fibroblasts express the same cell surface markers as MSCs (positive for CD73, CD90 and CD105 and negative for CD14, CD34, CD45, CD19 and HLA-DR) [24]. In this study, we have tested whether MSC mitochondria are comparable to fibroblast mitochondria. We find that MMP of fibroblasts and MSCs are comparable. However, MSC's mitochondria produce higher ATP content and OCR than fibroblast's mitochondria. The results support the concept that fibroblasts are in fact aged MSCs and that the two cells are the same [24]. It is reasonable that mitochondria of MSCs produce more ATP than mitochondria of the aged MSCs, fibroblasts.

In summary, mitochondria of fresh and cryopreserved fibroblasts and MSCs are comparable. Cryopreserved fibroblast and MSCs are alternative mitochondrial donors for MOT to fresh cells. In addition, MSCs could provide higher ATP-produced mitochondria than fibroblasts.

Acknowledgements

This work was supported by the State of South Carolina Carroll A. Campbell, Jr. Alzheimer's Research Initiative (Greenwood Genetics Center), 1I01BX006259-01A1 MERIT Award (MSK), and 5IK6BX005239-05 Senior Research Career Scientist Award (MSK).

Conflicts of Interest

Xianpeng Jiang, Catherine C. Baucom and Brent Segal have research support from MitoSense Inc. and also serve on the Board of Directors of MitoSense Inc.

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