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Extracellular Vesicles from Mesenchymal Stromal Cells (imEVs) Improve Cold Preservation of Isolated Mitochondria

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Abstract

Mitochondrial organelle transplantation (MOT) is an innovative strategy for the treatment of mitochondrial dysfunction such as cardiac ischemic reperfusion injuries, Parkinson's diseases, brain and spinal cord injuries, and amyotrophic lateral sclerosis (ALS). However, one of the major challenges for widespread usage is a methodology for preservation of isolated mitochondria. Extracellular vesicles (EVs) are phospholipid bilayer-enclosed vesicles released from cells. EVs carry a cargo of proteins, nucleic acids, lipids, metabolites, and even organelles such as mitochondria. **Purpose**: To test if EVs enhance the stability of isolated mitochondria. **Methods**: We mixed isolated mitochondria of fibroblasts with EVs of mesenchymal stromal cells (imEVs) (9:1 in volume) and stored the mixture at 2˚C - 6˚C for different time periods. We measured morphology, mitochondrial membrane potential (MMP) and mitochondrial ATP content at 0, 2, 5 days. **Key findings**: After 2 days of storage, the mitochondria without imEVs lost approximate 70% MMP (RFU: 1822 ± 68), compared to the fresh mitochondria (RFU: 5458 ± 52) ($p < 0.01$). However, MMP of the mitochondria mixed with imEVs (RFU: 6786 ± 291) was even slightly higher than MMP of the fresh mitochondria (RFU: 5962 ± 222) ($p > 0.05$). In agreement with MMP, mitochondria without imEVs lost significant mitochondrial ATP content ($p < 0.01$), but the mitochondria with imEVs addition preserved at least 90% mitochondrial ATP ($p > 0.05$), after 2 days of cold storage, compared to fresh mitochondria. Microscopy showed that imEVs promoted aggregation of isolated mitochondria. **Summary:** The preliminary data showed that imEVs enhanced the stability of isolated mitochondria in cold storage.

Keywords

Mitochondria, Extracellular Vesicles, Mitochondrial Preservation, MOT, imEVs

1. Introduction

In eukaryotic cells, mitochondria are well known as the powerhouse which generates ATP by oxidative phosphorylation (OXPHOS). They also play an important role in synthesis of iron-sulfur clusters and heme, β-oxidation of fatty acids, the urea cycle, and homeostasis of calcium, iron and reactive oxygen species (ROS) [1] [2] [3]. Mitochondrial dysfunction plays an important role in many diseases such as cardiovascular disease, metabolic disease, neurodegenerative disease, etc. [3] [4] [5]. Traditional drugs or genes have difficulty entering specific sub-compartments of mitochondria. Moreover, the diverse nature of gene mutations among patients makes it impossible to develop one drug to correct mitochondrial dysfunction. In recent years, mitochondrial organelle transplantation (MOT) has shone a new light on therapeutic intervention that benefits neuronal survival and regeneration for neurodegenerative diseases, stroke and CNS injury [6] [7]. McCully *et al.* reported the first clinical trial of mitochondrial transplantation therapy. They performed an autologous mitochondrial transplantation for myocardial ischemia–reperfusion injury of pediatric patients who required extracorporeal membrane oxygenation (ECMO) [8]. Elliott *et al.* reported a case study that MOT significantly improved leg muscle strength and recovered all sensory sensations in a deteriorated ALS patient [6]. It has been reported that the isolated mitochondria lost significant activity when stored on ice for more than 1 hour, thus a rapid operation is essential during clinical trial [9]. Cold storage and cryopreservation of mitochondria haven't been successful for long term storage, leading to the decrease in respiratory capacity and damage of mitochondrial membrane structure over time [10]. Thus, the preservation of mitochondria is a significant challenge for MOT.

Membranous extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies (ApoBDs), facilitate intercellular communication following their release from donor cells and subsequent internalization into recipient cells. EVs can encapsulate small portions of the subjacent cytosol and create a heterogeneous population of phospholipid-walled vesicles. ApoBDs are the largest EVs in size and contain a wide variety of cellular components: micronuclei, chromatin remnants, cytosol portions, degraded proteins, DNA fragments, or even intact organelles [11] [12]. MSC-EV-mediated mitochondrial transfer from the EVs to injured alveolar epithelia and endothelia has been reported. In the acute respiratory distress syndrome (ARDS) models, MSC-EVs improve alveolar–capillary barrier properties through restoration of mitochondrial functions at least partially via mitochondrial transfer [13]. EVs can be stored at −20˚C

without losing the efficacy of inner cellular components including mitochondria. We hypothesize that EVs matrix could preserve the function of cellular components and enhance the stability of isolated mitochondria.

We mixed the EVs of mesenchymal stromal cells (imEVs) with isolated mitochondria of fibroblasts, and stored the mixture at 2˚C - 6˚C for different times. We measured morphology, mitochondrial membrane potential (MMP) and mitochondrial ATP content. The preliminary data showed that imEVs prolong the survival of isolated mitochondria in cold storage.

2. Materials and Methods

2.1. Isolation, Primary Culture and Cryopreservation of Human Fibroblasts

Collection of human skin tissue was approved by Western IRB (Protocol#20203104). All tissue pieces were digested by 3% collagenase type 3 (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37˚C for 5 hours in a sterile stirring chamber. The liquid was transferred into a sterile 50 ml centrifuge tube and centrifuged for 5 minutes at 400 g. Supernatant was removed. Cell pellet was resuspended in 30 ml pre-warmed (37˚C) complete alpha minimum essential media (alpha MEM) (GIBCO, Carlsbad, CA, USA) containing 5% human platelet lysate (HPL) (Biological Industries, Cromwell, CT, USA) and 0.05 mg/ml gentamicin (GIBCO, Carlsbad, CA, USA). Cells was cultured at 37˚C in 5% CO_2 incubator overnight. The medium and floating cells was aspirated next day. 30ml fresh medium was added to the flask. The flask was returned to the incubator for culture with medium refresh every 3 - 4 days. When cell growth was 80% confluent, cells were sub-cultured to new flasks by 1:10 dilution. The primary fibroblasts of second and third passages were collected, re-suspended in Nutrifreez D10 cryopreservation medium (Biological Industries, Cromwell, CT, USA), aliquoted to 1 ml containing 1×10^6 cells in cryovials, frozen and stored in liquid nitrogen.

2.2. Fibroblast Expansion

Human primary fibroblasts were established and stored in liquid nitrogen [14]. 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). 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.

2.3. Mesenchymal Stromal Cell (MSC) Culture

BM-MSCs were cultured on laminin-521 coated plates in StemMACS MSC Expansion Medium (Miltenyi Biotec, Germany) at 37˚C and 5% CO_2. Laminin-521 was purchased from Biolamina Ab, Sweden and the cell culture plates were coated according to the manufacturer's instructions. For production of EVs,

BM-MSCs were cultured until approximately 80% confluency and carefully washed twice with PBS. After that, serum-free Opti-MEM™ medium (GIBCO, Carlsbad, CA, USA) was added and the cells were incubated for 48 hours at 37˚C and 5% CO_2. Then, the conditioned medium was collected, and centrifuged first for 5 minutes at 700 g to remove living cells and a second spin for 100 minutes at 250 g to remove cellular debris. The cells that remain are cultured for 24 hours in the complete culturing medium. Another production round was made as described above.

2.4. Isolation of EVs

The centrifuged conditioned medium was sterile filtered and concentrated using tangential flow filtration (TFF), KR2i TFF system (SpectrumLabs). For that, a modified polyethersulfone (mPES) hollow fiber filter with 300 kDa membrane pore size was used (MidiKros, 370 cm^2 surface area, SpectrumLabs). The flow rate was set at 100 ml/min. At first, the conditioned medium was concentrated to a volume of 75 ml in the container (100 ml total volume in the system) and, then, dialyzed with 1 liter of sterile filtered PBS. After the dialysis, the sample was concentrated to 30 ml and sterilized using a 0.22 μm filter. Finally, the sample was concentrated to a volume of 1 - 2 ml using a 10 ml 10 kDa MWCO spin filter (Amicon).

2.5. Western Blotting

The samples were mixed with Laemmli Sample Buffer (Bio-Rad) under reducing conditions and warmed at 95˚ for 10 min. Then, 4% - 12% gradient gels were used for SDS electrophoresis and the proteins were transferred to polyvinylidene fluoride membranes using iBlot 2 (Invitrogen) according to the manufacturer's instructions. The membranes were first hybridized with the antibody of interest and, then, with the corresponding HRP-conjugated secondary antibodies. For visualization, chemoluminescent HRP-substrate from Amersham Biosciences was used and the figures were made using ChemiDoc imaging system (Bio-Rad).

2.6. Transmission Electron Microscopy

A 5 μl drop of the sample was placed on a formvar and carbon coated 200-mesh copper grid. The excess solution was removed by blotting with filter paper. The sample was then directly contrasted with 2% uranyl acetate. Excess of uranyl acetate was removed by blotting on filter paper. The contrasting step was repeated twice. Dried grids were examined by Tecnai™ G2 Spirit BioTwin transmission electron microscope (Thermo Fisher/FEI) at 80 kV with an ORIUS SC200 CCD camera and Gatan Digital Micrograph software (both from Gatan Inc.).

2.7. Detection of Mitochondrial Genes by Polymerase Chain Reaction (PCR)

Mitochondrially encoded cytochrome c oxidase I (MT-CO1) and mitochondrially encoded NADH dehydrogenase 1 (MT-ND1) in the samples of imEVs

were detected by PCR [14]. PCR was performed in a total 25 µl volume including 2 µl of PCR Master mix (Thermofisher Scientific, Waltham, MA, USA), 0.5 µl of 100 µM forward and reverse primers and 1 µl of heat-denatured imEVs samples. The primers for MT-CO1 amplification were 5'-CTAGCAGGTGTCTCCTCTATCT-3' and 5'-GCTCGTGTGTCTACGTCTATTC-3'. The primers for MT-ND1 were 5'-CCTTCGCTGACGCCATAAA-3' and 5'-GGTCTCTGCTAGTGTGGAGATA-3'. β-actin (ACTB) was used as endogenous gene control. The primers for ACTB amplification were 5'-CCTTTCTCACTGGTTCTCTCTTC-3' and 5'-CGTAGCACAGCTTCTCCTTAAT-3'. The amplification procedure included initial DNA denaturing at 95˚C for 3 minutes, then 35 cycles of denaturing 30 seconds at 95˚C, primer annealing 30 seconds at 50˚C and 60 seconds of extension at 72˚C, and final extension of 10 minutes at 72˚C in a T100 Thermal Cycle (Bio-Rad, Hercules, CA, USA). PCR products were run on a 2% agarose gel and imaged by ethidium bromide fluorescence.

2.8. Isolation of Mitochondria

Mitochondrial isolation follows a previously described protocol [14]. All reagents were sterile. Fibroblasts 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. The mitochondrial pellet was re-suspended with 240 mM sucrose mitochondrial respiration buffer (MRB) (Sigma Aldrich, St. Louis, MO, USA). We mixed the isolated mitochondria of fibroblasts with imEVs (9:1 in volume) and stored the mixture at 2˚C - 6˚C for 2 and 5 days. MMP and mitochondrial ATP content were measured and compared to them of fresh mitochondria (0 day).

2.9. Measurement of MMP

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 (rosamine- or cyCarbocyanine-based probes) have been used to stain mitochondria and monitor mitochondrial potential [15]. The stock solution of JC-1 is added to mitochondrial suspension to a final concentration 1 µg/ml. The mixture is incubated for 10 minutes at room temperature. For a control assay, stock solution of valinomycin is added to mitochondrial sample to a final concentration of 10 µg/ml. The mitochondrial sample containing valinomycin is kept on ice for 10 minutes to allow complete dissipation of the MMP.

The valinomycin-treated mitochondria are stained with JC-1 dye for 10 minutes at room temperature. Red fluorescent J-aggregates in intact mitochondria can be observed under fluorescent microscope. The relative fluorescence units (RFU) can be read in multiple plate fluorimeter using end-point method with the setting of Ex/Em: 490 nm/590nm.

2.10. Measurement of Mitochondrial ATP Content

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

2.11. Statistical Analysis

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

3. Results

3.1. Characterization of imEVs

imEVs were characterized according to the International Society for Extracellular Vesicles guidelines. NanoSight 3000 device and transmission electron microscopy revealed the characteristic sizes and shapes for exosomes. The EV samples expressed positive markers of EVs (CD63, CD81, and TSG101) but negative marker of EVs (Calnexin) by using West-blotting.

3.2. Viable Mitochondria in imEVs

imEVs were shipped on dry ice and stored at −20˚C after arrival. At 7 days post-freezing, the imEVs were thawed at 4˚C and stained with JC-1. The mitochondrial genes in the imEVs were determined by PCR. We found intact mitochondria that actively concentrated JC-1 dye in the imEVs sample (**Figure 1**). Mitochondrial genes of MT-ND1 and MT-CO1 in the imEVs were amplified by PCR (**Figure 2**). The JC-1 staining and PCR confirmed the imEVs contained viable mitochondria. The viable mitochondria maintained MMP even though the imEVs went through freezing-thawing cycles. The viable mitochondria may be apoptotic bodies or free mitochondria in the imEVs solution. The results suggest that imEVs may enhance the stability of extracellular mitochondria.

3.3. imEVs Stabilize the Isolated Mitochondrion's MMP in Cold Storage

The mitochondria and the mixture of mitochondria with imEVs were stored at

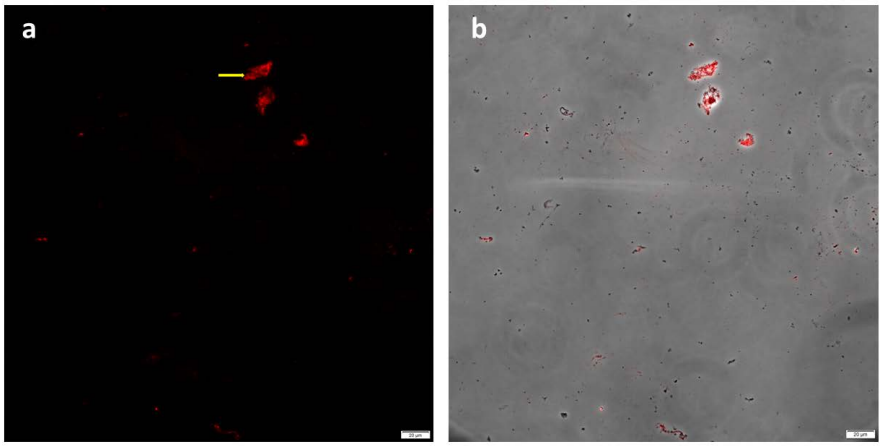

Figure 1. JC-1-stained mitochondria in imEVs. (a) fluorescent; (b) phase contrast + fluorescent; yellow arrow: J-aggregates of mitochondria. The details are seen in the Materials and Methods.

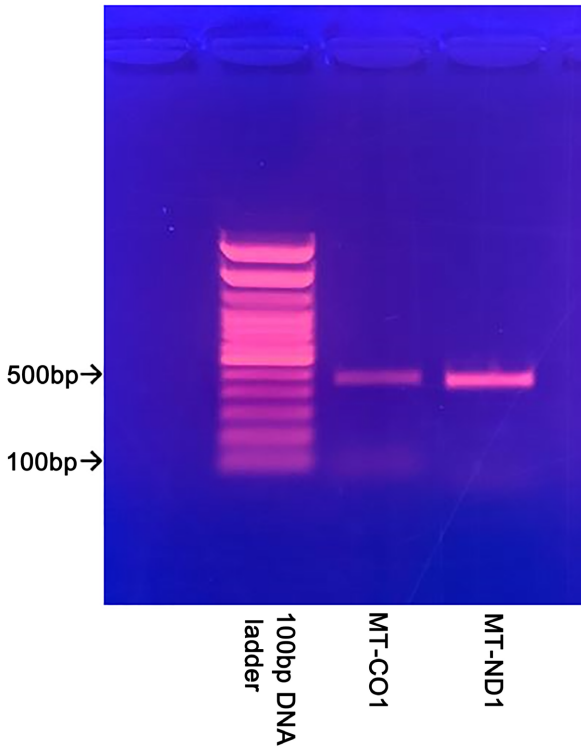

Figure 2. Mitochondrial genes in imEVs. Genes were amplified by PCR. The details are seen in the Materials and Methods.

2˚C - 6˚C for 2 and 5 days. Microscopy showed that the imEVs stimulated aggregation of the isolated mitochondria and maintained MMP, compared to the mitochondria without imEVs (**Figure 3**). MMP was measured by a multiple plate fluorimeter using end-point method with the setting of Ex/Em: 490 nm/590nm. The MMP was compared to the MMP of fresh mitochondria (at 0 day). The isolated mitochondria stored at 2˚C - 6˚C for 2 days lost significant MMP (RFU 5458 ± 52 at 0 day vs. 1822 ± 68 at 2 days, $p < 0.01$). The MMP at 2

days was approximately 33% of MMP at 0 day. However, the mitochondria mixed with imEVs maintained and even slightly increased the MMP after 2 days of storage at 2˚C - 6˚C (RFU 5962 ± 222 at 0 day vs. 6786 ± 291 at 2 days, $p > 0.05$). Mitochondria with imEVs have significantly higher MMP than the mitochondria without imEVs (6786 ± 291 vs. 1822 ± 68, $p < 0.01$) after 2 days of preservation at 2˚C - 6˚C (Table 1). After 5 days, both the mitochondrial without and with imEVs lost 73% and 70% of MMP ($p < 0.01$, $P < 0.01$, compared to fresh mitochondria), respectively (Table 1, Figure 4). The preliminary results showed that imEVs prolonged the survival of isolated mitochondria in cold storage at least 2 days.

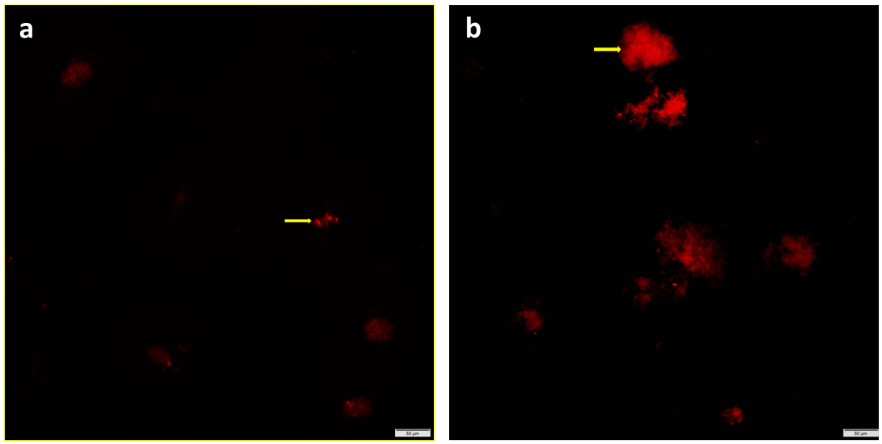

Figure 3. imEVs stimulated mitochondrial aggregation and stabilized MMP. The isolated mitochondria were stained with JC-1 after 2 days of storage at 2˚C - 6˚C. (a) mitochondria without imEVs; (b) mitochondria + imEVs (9:1 in volume); yellow arrow: J-aggregates of mitochondria.

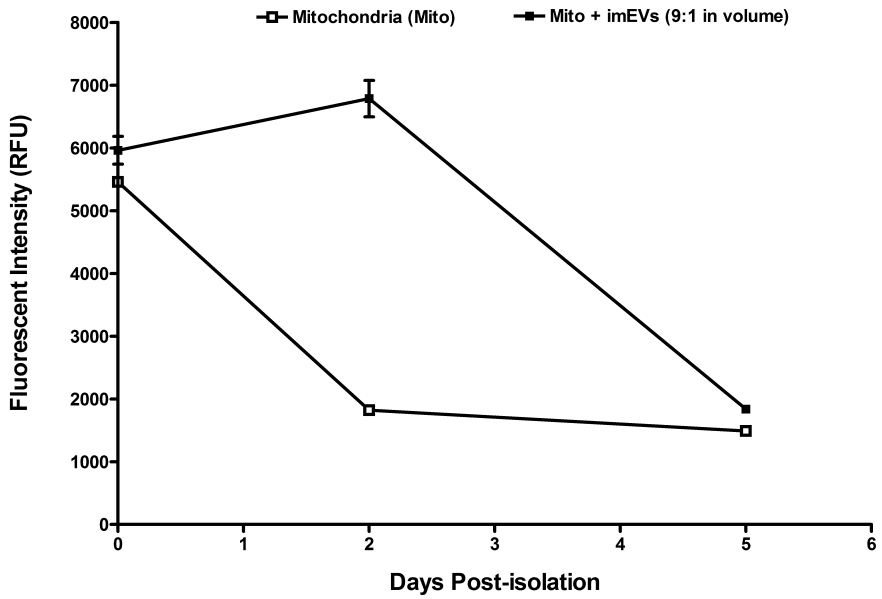

Figure 4. EVs stabilized MMP of isolated mitochondria. Mitochondria were kept at 2˚C - 6˚C.

Table 1. imEVs preserved MMP of isolated mitochondria. The mitochondria were stained with JC-1. RFU was measured by multiple plate fluorimeter using end-point method with the setting of Ex/Em: 490 nm/590nm.

Time Post-isolation	0 day	2 days	5 days
Mitochondria (without imEVs)	5458 ± 52 (3)[1]	1822 ± 68 (3) ($p < 0.01$)[2]	1490 ± 12 (3) ($p < 0.01$)[2]
Mitochondria + imEVs (9:1 in volume)	5962 ± 222 (3) ($p > 0.05$)[3]	6786 ± 291 (3) ($p > 0.05$)[2] ($p < 0.01$)[3]	1838 ± 37 (3) ($p < 0.01$)[2] ($p > 0.05$)[3]

[1]Relative fluorescence units (RFU), mean ± standard deviation (SD) (N); [2]Compared to fresh mitochondria (0 day); [3]Compared to the Mitochondria (without imEVs).

3.4. imEVs Preserve ATP Content of the Isolated Mitochondria

In agreement with the MMP, the isolated mitochondria lost approximately 40% ATP content after 2 days cold storage at 2˚C - 6˚C, compared to fresh isolated mitochondria (0 day) ($p < 0.01$). In contrast, the mitochondria mixed with imEVs maintained approximately 90% ATP content, compared to fresh mitochondria ($p > 0.05$) (Table 2). Mitochondria with imEVs have significantly higher ATP content than the mitochondria without imEVs (90 ± 8 vs. 60 ± 9, $p < 0.05$) (Table 2). After 5 days of cold storage, mitochondria in both solutions lost significant ATP content ($p < 0.01$, $p < 0.01$, respectively, compared to fresh mitochondria) (Table 2, Figure 5).

4. Discussion

It has been reported that the isolated mitochondria lost significant activity when stored on ice for more than 1 hour [9]. Thus, clinical applications of mitochondrial transplantation would benefit from improved storage. The development of mitochondrial storage for an extended period is a very important issue [10]. EVs are phospholipid bilayer-enclosed vesicles naturally released from all cell types. EVs carry a cargo of proteins, nucleic acids, lipids, metabolites, and even organelles such as mitochondria from the parent cells [16]. We found viable mitochondria in our imEVs using JC-1 staining and PCR assay even though the imEVs went through freezing-thawing cycles. The findings suggested that EVs preserved the viability of mitochondria that were enclosed in EVs (apoptotic bodies) or outside EVs (mitochondrial contaminants). To confirm the findings, we mixed isolated mitochondria of fibroblasts with imEVs of MSCs as the 9:1 ratio and stored the mitochondrial solutions at 2˚C - 6˚C. After 2 days of storage, the isolated mitochondria without imEVs lost significant MMP and ATP content, but the mitochondria with imEVs addition maintained the almost same of MMP and ATP content as fresh mitochondria. Moreover, imEVs promoted mitochondrial aggregation that might be one of mechanisms to enhance mitochondrial viability. The results suggest that EVs can extend the viability of mitochondria in cold storage at least 2 days.

Further study remains to be done as the following: 1) ATP production and

Table 2. imEVs enhance the stability of mitochondrial ATP. Mitochondrial ATP content was calculated as the percentage of the fresh isolated mitochondria.

Time Post-isolation	0 day	2 days	5 days
Mitochondria without imEVs	100 ± 0 (3)[1]	60 ± 9 (3) ($p < 0.01$)[2]	30 ± 6 (3) ($p < 0.01$)[2]
Mitochondria + imEVs (9:1 in volume)	100 ± 0 (3) ($p > 0.05$)[3]	90 ± 8 (3) ($p > 0.05$)[2] ($p < 0.05$)[3]	42 ± 7 (3) ($p < 0.01$)[2] ($p > 0.05$)[3]

[1]Mitochondrial ATP content, % fresh mitochondria (0 day), mean ± standard deviation (SD) (N); [2]Compared to fresh mitochondria (0 day); [3]Compared to the Mitochondria without imEVs.

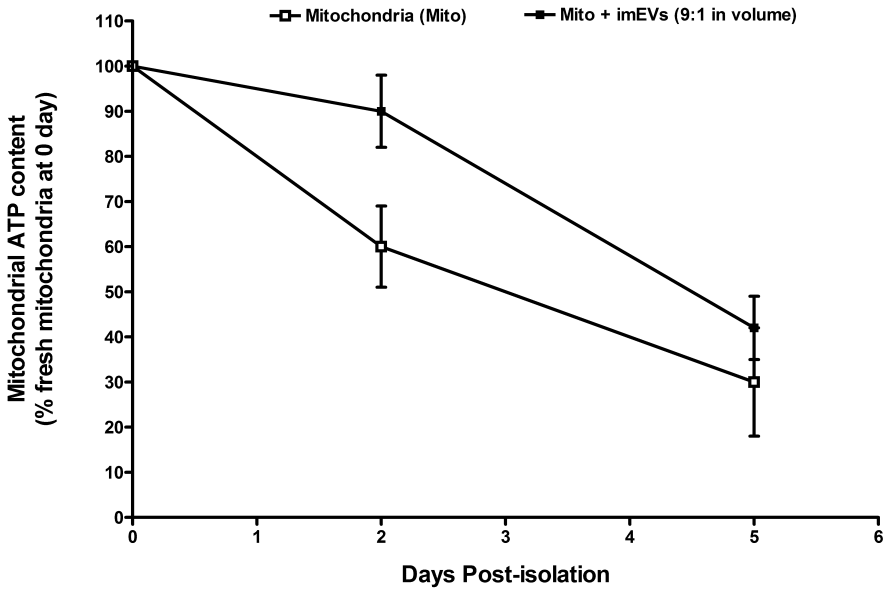

Figure 5. imEVs stabilizes mitochondrial ATP. The mitochondria were stored at 2˚C - 6˚C. Mitochondrial ATP content was calculated as the percentage of the fresh isolated mitochondria.

oxygen consumption of mitochondria with and without imEVs using Seahorse system; 2) Are mitochondria packed into imEVs or only trapped in the matrix of imEVs? 3) What the optimal ratio of mitochondria to imEVs to extend the survival of the mitochondria? 4) Do imEVs change the ability of mitochondrial transplant to cells? Our current results are preliminary but provide a potential new preservation method to extend the life of isolated mitochondria for MOT application.

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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.. Dr. Sergey Rodin and Dr. Karl-Henrik Grinnemo are co-owners of the company AVulotion AB.

References

[1] Murphy, E., Ardehali, H., Balaban, R.S., DiLisa, F., DornII, G.W., *et al.* (2016) Mitochondrial Function, Biology, and Role in Disease. *Circulation Research*, **118**, 1960-1991. https://doi.org/10.1161/RES.0000000000000104.

[2] Braymer, J.J. and Lill, R. (2017) Iron-Sulfur Cluster Biogenesis and Trafficking in Mitochondria. *Journal of Biological Chemistry*, **292**, 12754-12763. https://doi.org/10.1074/jbc.R117.787101

[3] Herst, P.M., Rowe, M.R., Carson, G.M. and Berridge, M.V. (2017) Functional Mitochondria in Health and Disease. *Frontiers in Endocrinology*, **9**, 1-16. https://doi.org/10.3389/fendo.2017.00296.

[4] Smith, E.F., Shaw, P.J. and De Vos, K.J. (2019) The Role of Mitochondria in Amyotrophic Lateral Sclerosis. *Neuroscience Letters*, **710**, Article ID: 132933. https://doi.org/10.1016/j.neulet.2017.06.052

[5] Federico, A., Cardaioli, E., Pozzo, P.D., Formichi, P., Gallus, G.N. and Radi, E. (2012) Mitochondria, Oxidative Stress and Neurodegeneration. *Journal of the Neurological Sciences*, **322**, 254-262. https://doi.org/10.1016/j.jns.2012.05.030

[6] Elliott, R. and Jiang, X. (2020) Neurodegeneration and Mitochondria Organelle Transplantation: "A Technology That Proof of Principle Suggest Is Ready for Prime Time". *Neuroscience and Medicine*, **11**, 108-118. https://doi.org/10.4236/nm.2020.114013

[7] Norat, P., Soldozy, S., Sokolowski, J.D., Gorick, C.M., *et al.* (2020) Mitochondrial Dysfunction in Neurological Disorders: Exploring Mitochondrial Transplantation. *npj Regenerative Medicine*, **5**, Article No. 22. https://doi.org/10.1038/s41536-020-00107-x

[8] Emani, S.M., Piekarski, B.L., Harrild, D., Del Nido, P.J. and McCully, J.D. (2017) Autologous Mitochondrial Transplantation for Dysfunction after Ischemia-Reperfusion Injury. *The Journal of Thoracic and Cardiovascular Surgery*, **154**, 286-289. https://doi.org/10.1016/j.jtcvs.2017.02.018

[9] McCully, J.D., Cowan, D.B., Emani, S.M. and Del Nido, P.J. (2017) Mitochondrial Transplantation: From Animal Models to Clinical Use in Humans. *Mitochondrion*, **34**, 127-134. https://doi.org/10.1016/j.mito.2017.03.004

[10] Yamada, Y., Momo Ito, M., Arai, M., Hibino, M., Tsujioka, T. and Harashima, H. (2020) Challenges in Promoting Mitochondrial Transplantation Therapy. *International Journal of Molecular Sciences*, **21**, Article 6365. https://doi.org/10.3390/ijms21176365

[11] Murphy, D.E., de Jong, O.G., Brouwer, M., Wood, M.J., Lavieu, G., Schiffelers, R.M. and Pieter Vader, P. (2019) Extracellular Vesicle-Based Therapeutics: Natural versus Engineered Targeting and Trafficking. *Experimental & Molecular Medicine*, **51**, 1-12. https://doi.org/10.1038/s12276-019-0223-5

[12] Battistelli, M. and Falcieri, E. (2020). Apoptotic Bodies: Particular Extracellular Vesicles Involved in Intercellular Communication. *Biology*, **9**, Article 21. https://doi.org/10.3390/biology9010021

[13] Silva, J.D., Yue Su, Calfee, C.S., Delucchi, K.L., Weiss, D., McAuley, D.F., O'Kane, C. and Krasnodembskaya, A.D. (2021) Mesenchymal Stromal Cell Extracellular Vesicles Rescue Mitochondrial Dysfunction and Improve Barrier Integrity in Clinically Relevant Models of ARDS. *European Respiratory Journal*, **58**, Article ID: 2002978. https://doi.org/10.1183/13993003.02978-2020

[14] Jiang, X. , Baucom, C. and Elliott, R. (2020) Mitochondria Dynamically Transplant into Cells in Vitro and in Mice and Rescue Aerobic Respiration of Mitochondrial DNA-Depleted Motor Neuron NSC-34. *Journal of Biomedical Science and Engineering*, **13**, 203-221. https://doi.org/10.4236/jbise.2020.139019

[15] Sivandzade, F., Bhalerao, A. and Cucullo, L. (2019) Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dyeas a Sensitive Fluorescent Probe. *Bio Protocol*, **9**, e3128. https://doi.org/10.21769/BioProtoc.3128

[16] Elsharkasy, O.M., Nordin, J.Z., Hagey, D.W., de Jong, O.G., Schiffelers, R.M., Andaloussi, S.E. and Vader, P. (2020) Extracellular Vesicles as Drug Delivery Systems: Why and How? *Advanced Drug Delivery Reviews*, **159**, 332-343. https://doi.org/10.1016/j.addr.2020.04.004.