Centrifugation: A New Frontier for Mitochondrial Transfer

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ABSTRACT

The mitochondrion is most notably known as the powerhouse of the cell, but malfunctions of this organelle have underscored its importance in a myriad of aspects. Mitochondrial disease has been linked to cancer,[1] aging, and neurodegenerative illnesses,[2] like dementia. Replacement of the mitochondrion and its genetic material has been the basis of mitochondrial therapy, but studies replacing mitochondrial DNA (mtDNA) have displayed reversion to parental mtDNA. Little is known about the mechanisms underpinning this phenomenon, with it possibly occurring exclusively in humans.[3] Consequently, the transfer of whole mitochondria appears a more viable option: it's proven to be successfully moved between cells.[4-9] However, the current methods for horizontal transfer have failed to address maintaining mitochondrial quality,[5] preserving function over longer periods of time, and the conflicting results of uptake.[10,11] Contrastly, centrifugation has demonstrated to be a simpler and efficient method for mitochondrial transfer and capable of preserving quality over longer periods.[5]

SUMMARY

The objective of this experimental study is to assess the effectiveness of centrifugation for mitochondrial transfer between cells in comparison to existing methods: co-culturing and microinjection. Currently, there are no methods of successful mitochondrial transfer that can preserve mitochondrial quality while sustaining function over long periods of time.[12]

The mitochondrion has its own genetic material— 37 genes specifically— and thirteen of which are involved in the synthesis of adenosine triphosphate (ATP), the cell's main source of energy, during cellular respiration. The remaining genes are instrumental in the process of assembling proteins.[13] As mtDNA is regularly exposed to harmful products of cellular respiration, it's more susceptible to damage than nuclear DNA.[14] Genetic mutations in this organelle can give rise to Mitochondrial disease, a condition affecting 1 in 5000 adults.[15] This general term encompasses a myriad of conditions indiscriminately affecting organ systems. Depending on which cells are affected, Dr. Falk, a physician from the Children's Hospital of Philadelphia, states patients can experience up to 16 different symptoms ranging from mild to fatal conditions such as strokes, muscle weakness, liver failure, and respiratory problems.[16] Currently, some mitochondrial transfer techniques involve genetic modification called Mitochondrial Replacement Therapy (MRT). However, this method is prohibited under federal law. Consequently, the horizontal transfer of the mitochondria is solely at the forefront of combating Mitochondrial disease, but established techniques are unable to preserve mitochondrial quality over a long time and need improvements. With that said, centrifugation has the potential to advance whole mitochondrial transfer to clinical applications and ultimately pioneer treatment for Mitochondrial disease.

CURRENT SOLUTIONS

Current methods of whole mitochondrial transfer assessed here are co-culture and microinjection. Co-culture involves a controlled environment of liquid media (cell culture) in which two or more cell types grow. Scientists have used this technique in mitochondrial transfer by pairing a cell with a functional mitochondrion and one without. In one study, bone marrow stem cells and p^0 cells (cells deprived of mtDNA) were co-cultured and mitochondrial transfer from the stem cells to p^0 cells was successful. However, analyses of p^0 mtDNA displayed several gene sequences absent while some cells had acquired a functional mitochondrion.[17] In this and other studies, the p^0 cells were treated with ethidium bromide, a molecule that binds to DNA, considerably changing its structure and interfering with DNA replication and repair. In another recent study using co-culture, it improved mitochondrial function; functional gain of the new mitochondria was assessed via resazurin-based assays (dye-based) which track the reduction of living cells.[8] However, the internalized mitochondria disappeared within a week, indicative of limited functionality. Ultimately, although co-culture enables successful transfer, it is unable to sustain mitochondrial functionality over long durations.

Another method that has been investigated is microinjection, a protocol involving the injection of the mitochondria into the cell. Studies using this method have been done on gametes (sex cells). In one experiment, 217 fertilized cells, or zygotes, were injected with foreign mitochondria. Only 67 zygotes survived and 37 continued development.[9] Twenty-three of the cells that did continue to develop showed detectable amounts of foreign mtDNA. Evidently, microinjection has demonstrated to impede development and lower cell viability. Another study also injected a control group of cells with buffer. While microinjection of buffer had 98.8% survival, cells injected with mitochondria had only 56.5% survival.[18] In this experiment, the

mitochondria were separated using differential centrifugation, whereby cellular components are separated by mass. The mitochondrial suspension was then injected using a micromanipulator, a device used to physically work with microscopic samples; mtDNA was detected using PCR analysis. The lower rate of cell development shown in this study can be attributed to the use of somatic (non-sex) mitochondria in sex cells: the mitochondria was possibly detected as foreign within the cell, inhibiting development. On the other hand, another explanation can be that gametes are not as receptive to mitochondria as somatic cells.

Nonetheless, from the results of these aforementioned studies, neither method can preserve mitochondrial quality over time, leading to lower cell viability.

PROPOSED SOLUTION

In lieu of co-culture and microinjection, I suggest another method that has been investigated drastically less but has the potential to overcome the insufficiencies of its counterparts— centrifugation. Research scientist, Pavel Moroz theorized that centrifugal force can enable organelle transfer by forcing the mitochondria into targeted calls.[19] One study on this phenomenon focuses on the importance of both cell membrane permeability and centrifugal force in enabling mitochondrial uptake. The group isolated mitochondria from human umbilical cord-deprived stem cells (UC-MSCs) and spun desired amounts of mitochondria and targeted cells together at 1,500 x g for 5 minutes.[5] Targeted cells, which included cancer cells, were primed with PF-68, a substance that inserts itself into the plasma membrane, thus making it more permeable.[20] Amounts of PF-68 had a direct correlation with the amount of mitochondria entering targeted cells. Most notably, the study indicated that if the targeted cells were primed with PF-68, but no centrifugal force was imposed on it and the isolated mitochondria, then

horizontal transfer did not occur. Additionally, centrifugation yielded high mtDNA numbers than co-culture (see Appendix A).[5]

With that said, in my proposed study, I plan to replicate this experimental design including the component of executing co-culture as a basis of comparison. Mitochondria from mesenchymal stem cells (MSCs) will be isolated using differential centrifugation and then transferred into PC12 cells (a cell line derived from rats which has a phenotype similar to sympathetic ganglion neurons).[21] These specific cells were chosen because MSCs make up sympathetic nerves, and their malfunctions are causative factors of stroke in rats, a condition that can occur from Mitochondrial disease in humans.[22] Using these cell types will have direct implications on stroke and ultimately, Mitochondrial disease. The PC12 cells will be treated with varying amounts of ethidium bromide to deplete mtDNA; afterwards, they'll be primed with PF-68 and spun with the isolated mitochondria at 1,500 x g for 5 minutes. In this proposal, MSCs and PC12 cells will also be placed in a co-culture to assess mitochondrial uptake in comparison to centrifugation. PC12 cells will be grown in a medium containing pyruvate and uridine, an environment permissive for growth of cells devoid of mtDNA before being co-cultured with MSCs.[23]

To ascertain the presence of mitochondria in both procedures of co-culture and centrifugation, flow cytometry will be used: a technique by which a sample of cells are inserted and subjected to light beams; the scattered light correlates with cells and its components.[24] In addition, an imaging method called confocal microscopy will be implemented to visually see the transferred mitochondria, and amounts of mtDNA will be detected using PCR analysis. However, since the presence of mitochondria is not solely indicative of this study's success, the amount of ATP produced by target cells must also be analyzed. To measure ATP content and

rate, a bioluminescent ATP determination assay will be utilized (see Appendix B). ATP content post-centrifugation and co-culture will be taken every 24 hours for one week and there will be three trials in total, taking approximately 3 weeks to complete this study. I will be recording and averaging the amount of ATP from mitochondria pre-isolation, PC12 cells post-centrifugation, and PC12 cells post-co-culture. Data gathered will be analyzed in R through an ANOVA Test, a statistical method that will denote how statistically significant differences between the three groups are.

OBSTACLES

Because the study of centrifugal force on organelle transfer has not yet been replicated, there is a possibility mitochondrial transfer will not occur at 1,500 x g, so this proposed study will have to experiment with different speeds and times. Another obstacle is maintaining a sterile environment to avoid contamination when culturing cells and isolating mitochondria.

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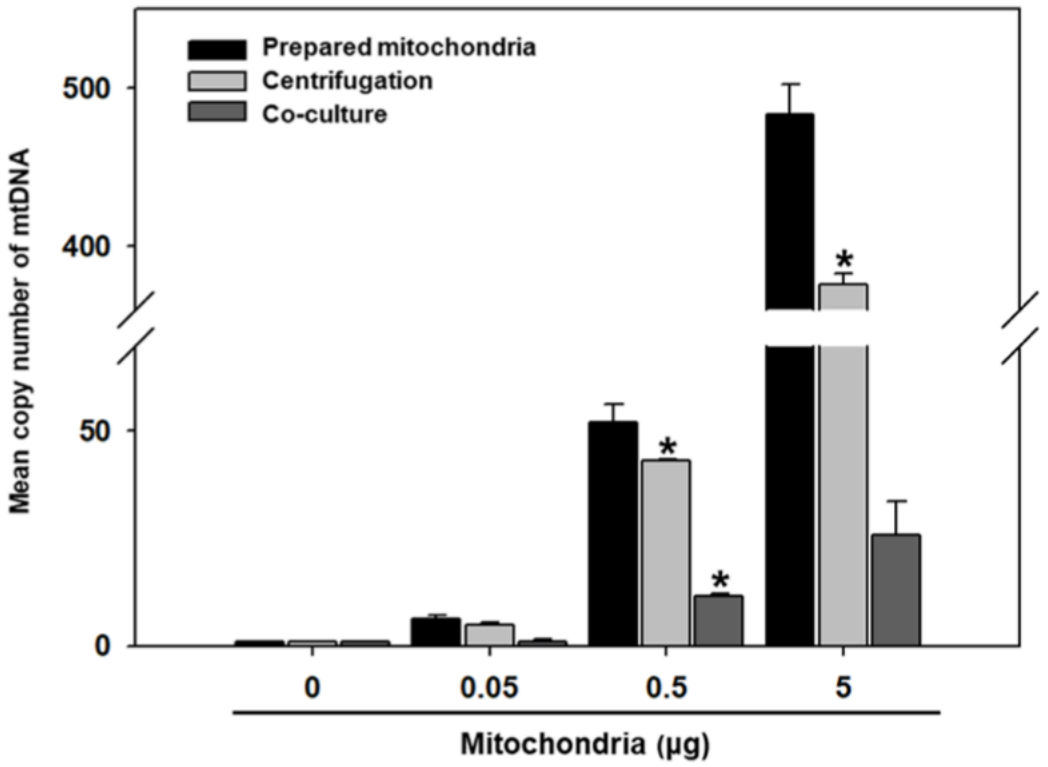
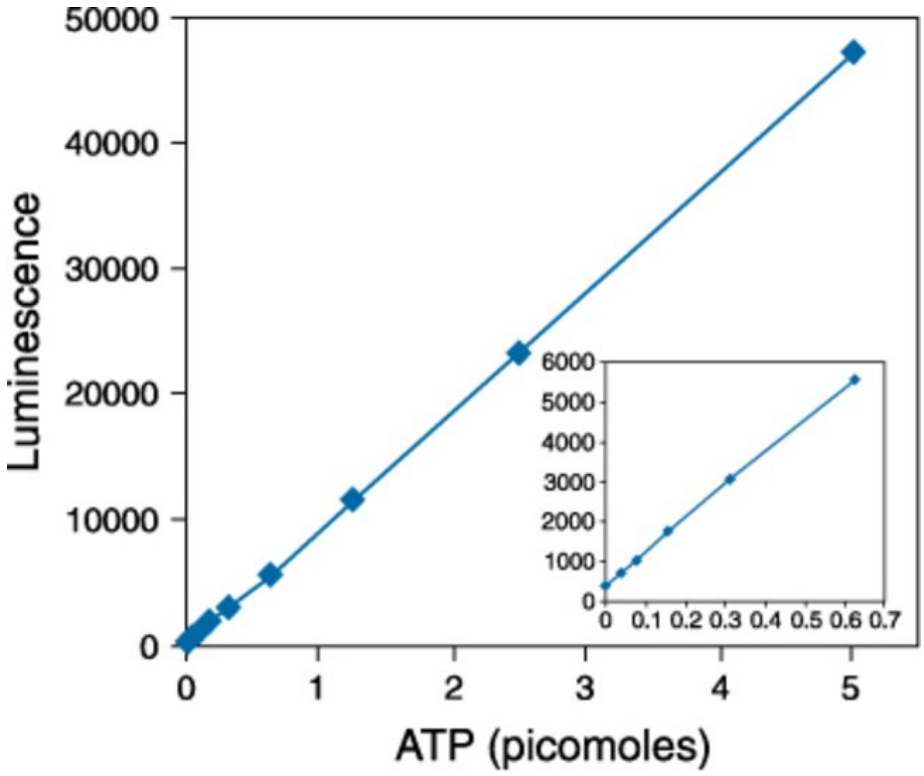
APPENDIX A



The above graph is of a PCR analysis of mean mtDNA of prepared isolated mitochondria, target cells post-centrifugation, and target cells after 1 day of co-culture from Delivery of exogenous mitochondria via centrifugation enhances cellular metabolic function (reference 5). This shows that centrifugation yields more average mtDNA transfer than co-culture.

APPENDIX B



Above is a sample graph from Thermo Fisher of the detection of ATP using their bioluminescence determination assay.