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Prospects of mitochondrial transplantation in clinical medicine: Aspirations and challenges

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Title: Prospects of mitochondrial transplantation in clinical medicine: aspirations and challenges

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Abstract:

Mitochondria, known as the powerhouse of the cell, are at the center of healthy physiology and provide cells with energy in the form of ATP. These unique organelles are also implicated in many pathological conditions affecting a variety of organs in various systems. Recently, mitochondrial transplantation, inspired by mitochondria's endosymbiotic origin, has been attempted as a potential biotherapy in mitigating a variety of pathological conditions. Mitochondrial transplantation consists of the process of isolation, transfer, and uptake of exogenous, intact mitochondria into damaged cells. Here, we discuss mitochondrial transplantation in the context of clinical medicine practiced in neurology, cardiology, pulmonary medicine, and oncology, among others. We outline the role of mitochondria in various pathologies and discuss the state-of-the-art research that potentially form the basis of new therapeutics for the treatment of a variety of diseases due to mitochondrial dysfunction. Lastly, we explore some of the challenges associated with mitochondrial transplantation that must be addressed before mitochondrial transplantation becomes a viable therapeutic option in clinical settings.

Mitochondrial dysfunction has been observed in many diseases spanning across multiple organ systems (Moro 2020, Simoes, Morciano et al. 2020, Bruni 2021, Popov 2021, Xu, Yu et al. 2021, Zhou, Ren et al. 2021). As well, these unique organelles, which are crucial for normal cellular function, can be the prime suspect for many pathological conditions. Mitochondria are primarily responsible for generating energy in the form of adenosine triphosphate (ATP) and are present in every cell in the human body except red blood cells. ATP generation by mitochondria produces little amounts of potentially destructive free radicals known as reactive oxygen species (ROS), which can be handled through various intracellular buffer systems. These radicals also play roles as second messengers in important cell signaling cascades (Moslen 1994, Sena and Chandel 2012, Zuo, Zhou et al. 2015) as well as cell signaling molecules for normal biologic processes (Auten and Davis 2009). However, accumulation of these byproducts of ATP production can harm the cell. While ATP production by mitochondria is essential for life, it may provoke damage to multiple cellular organelles and processes.

The endosymbiotic theory suggests that mitochondria were once primordial free-living single-cellular organisms, which may have been engulfed by larger, likely anaerobic cellular organisms to take advantage of their more efficient aerobic energy production (Margulis 1975). This adoption and billions of years of evolution likely led to eukaryotes' complexity. The evidence for this theory lies in that mitochondria contain their own DNA (mtDNA) in the form of circular DNA, similar to that found in bacteria, and likewise they contain two lipid bilayers (Shaw and Winge 2009). Mitochondria are also equipped with the intracellular machinery required to produce 13 of its mitochondrial proteins, but rely on the nuclear DNA to produce the other key proteins (Nass and Nass 1963).

In our opinion, it is likely due to this endosymbiotic origin that the internalization of mitochondria by recipient cells is possible. Intercellular transfer of mitochondria has been reported to naturally occur in humans as a normal repair mechanism, rescuing damaged cells (Hayakawa, Esposito et al. 2016, Levoux, Prola et al. 2021, Walters and Cox 2021). This physiologic phenomenon in a variety of ways inspired the current form of mitochondrial transplantation including autologous (isogenic), non-autologous (allogenic), and even xenogeneic transplantation (Jiang, Elliott et al. 2015, Ali Pour, Kenney et al. 2020, Doulamis, Guariento et al. 2020, Popov 2021). Given that mitochondrial dysfunction can be at the center of devastating pathological conditions, mitochondrial transfer, hereby referred to as mitochondrial transplantation, has exciting therapeutic potentials in modern medicine. Specifically, mitochondrial transplantation appears to be promising in treating ischemic diseases of the cardiovascular and central nervous systems, among others (Zhang, Ma et al. 2019, Popov 2021, Xu, Yu et al. 2021, Zhou, Ren et al. 2021). Mitochondrial transplantation also holds potential in therapies for pulmonary, and musculoskeletal pathologies and even for a variety of cancers (Elliott, Jiang et al. 2012, Zhou, Zhang et al. 2016, Jabbari, Roushandeh et al. 2020, Orfany, Arriola et al. 2020). Here we discuss the therapeutic potential of mitochondrial transplantation in various fields of medicine and outline some of the challenges that need to be addressed before mitochondrial transplantation can have a greater role in the clinical space.

Mitochondrial transplantation inspired by the natural phenomenon of mitochondrial transfer

Mitochondrial transplantation includes the process of isolation, transfer, and internalization of intact mitochondria into target cells or tissue (Figure 1). Although

most studies focus on the *in vitro* aspect of mitochondrial transplantation, in the context of clinical application, McCulley et al. (McCully, Cowan et al. 2017) suggests collection of isogenic or allogenic tissue samples (<0.1 g) from a subject's skeletal muscle (mainly the gastrocnemius muscle) through biopsy. The biopsy sample is then subjected to homogenization via an automated homogenizer, followed by a brief digestion and filtration process to break down the bilipid cellular membrane and free organelles. This is followed by centrifugation to concentrate mitochondria, which ultimately yields ~1 x 10^9 mitochondria per 0.1g.

While mitochondria are abundant in skeletal muscle, other sources should also be explored because it would involve a biopsy from a subject with healthy mitochondria or a healthy muscle from the same subject. For instance, platelets are also rich in mitochondria (Garcia-Souza and Oliveira 2014, Melchinger, Jain et al. 2019) and could be a better source of mitochondria due to their less invasive collection if: (1) it is determined that the source of mitochondria is not important in the context of success of transplantation and mitochondrial adoption by recipient cells/tissues and (2) the activity profile of platelets is similar to that of the recipient cells.

Several techniques have been explored for isolating mitochondria (Ali Pour, Hosseinian et al. 2021). While centrifugation has been used for decades to isolate mitochondria, the tradeoff between high purity or high yield has always been a challenge. As such fractionated mitochondrial magnetic separation (FMMS) may be a more clinically relevant isolation technique. FMMS is a method of isolating mitochondria which relies on labelling mitochondria with magnetically tagged antibodies, such as anti-TOM22, which targets the mitochondrial membrane 22 (Hubbard, Harwood et al. 2019). After a 30-minute coincubation, the labeled lysate

is passed through a column within a magnetic field, resulting in the magnetically tagged mitochondria to be easily elucidated from the column. Although more time consuming than traditional methods of isolating mitochondria, FMMS has been found to increase yield and purity of mitochondria when compared to traditional centrifugation methods (Hubbard, Harwood et al. 2019).

Several methods exist for inducing the internalization of allogenic mitochondria into cells, here we focus on the two clinically relevant models: direct injection and coincubation. Direct injection using needles has been attempted *in vitro* and *in vivo* (in animal models and in clinical settings) in which the isolated mitochondria are directly injected into the recipient cells/tissue (Masuzawa, Black et al. 2013, Kaza, Wamala et al. 2017, Guariento, Piekarski et al. 2021). Coincubation is also a clinically relevant method for mitochondrial transplantation in which isolated mitochondria are incubated alongside or in the vicinity of target recipient cells, allowing the cells to uptake the mitochondria similar to situations where distressed cells naturally uptake mitochondria (Kubat, Ulger et al. 2021, Xu, Yu et al. 2021). Shin et al. leveraged this method to deliver boluses of mitochondria to ischemic cardiac tissue using an intracoronary catheter, an appealing strategy to introduce mitochondria in a minimally-invasive manner to target tissue (Shin, Saeed et al. 2019). Both delivery methods, whether through direct injection or via a catheter, result in widespread mitochondrial uptake at the area at risk, as shown by PET scans and MRI using radioactively and magnetically tagged mitochondrial membranes, respectively (Cowan, Yao et al. 2016, Shin, Saeed et al. 2019). Although previous work demonstrates that up to 43.52% of mitochondria are successfully transplanted to the heart (Cowan, Yao et al. 2016), other studies report that only 3-7% of injected mitochondria are internalized into the cardiomyocytes

(Masuzawa, Black et al. 2013, Pacak, Preble et al. 2015). Thus, different techniques may have led to different yields for transplantation and there is currently no consensus in the field about that.

As for the mechanisms of mitochondrial internalization by the recipient cells, more studies are required to fully address this phenomenon and consequently devise more effective mitochondrial transplantation strategies. Previous studies by Kitani et al. (Kitani, Kami et al. 2014) and Kesner et al. (Kesner, Saada-Reich et al. 2016) suggest macropinocytosis involvement in internalization of mitochondria. In their study, Kesner et al. (Kesner, Saada-Reich et al. 2016) utilized transmission electron microscopy and observed mitochondria were engulfed by uterine endometrial cancer cells though cellular extensions and cell surface ruffling. A macropinocytosis inhibitor was used by both Kesner et al. and Kitani et al. to effectively inhibit mitochondrial internalization (Kitani, Kami et al. 2014, Kesner, Saada-Reich et al. 2016). More work was done to further investigate the mechanism for mitochondrial internalization, showing actin polymerization as a potential mediator of internalization. Pacak et al. (Pacak, Preble et al. 2015) blocked actin polymerization, caveola-dependent-clathrin endocytosis, tunneling nanotubes, and macro-pinocytosis via a variety of chemical inhibitors in cardiomyocytes. They showed that only Cytochalasin D, an actin polymerization inhibitor, decreased mitochondrial internalization, and accordingly suggested that actin-dependent endocytosis is responsible for internalization. Cowan et al. (Cowan, Yao et al. 2017) further studied the uptake of mitochondria through three-dimensional super-resolution microscopy and transmission electron microscopy. They found that allogenic mitochondria derived from fibroblasts or pluripotent stem cells injected into ischemic hearts were rapidly internalized through actin-dependent endocytosis within minutes and were

subsequently transported to endosomes and lysosomes. Although ultimately destined to fuse with lysosomes to create late-endosomes, it is their hypothesis that mitochondria are able to escape these endosomes and integrate with the cytoplasm. This hypothesis is supported by the recent work that shows transplanted mitochondria are rapidly transported into cardiomyocytes and escape pre-endosomes and late endosomes to fuse with the endogenous mitochondrial network (Cowan, Yao et al. 2017). In fact, it is reported that over 80% of mitochondria taken up by the cell are integrated into the cell instead of degrading in lysosomes (McCully, Del Nido et al. 2022). Patel et al. have argued that only Kesner's work on actin-dependent internalization is reliable, and that more studies are needed to further elucidate the exact mechanism (Patel, Rorbach et al. 2017). We assume that a variety of mechanisms individually or in combination are responsible for mitochondrial internalization.

Applications of Mitochondrial transplantation in treatment of neurological disorders

Neurons heavily depend on mitochondria for ATP production to fuel the processes required for neurotransmission (e.g., regulation of the sodium potassium ATPase pump, exocytosis/recycling of synaptic vesicles, and regulation of intracellular calcium concentrations) (Bruni 2021). Given that the brain consumes 20% of oxygen and 50% of glucose supplied through delivery from the vasculature, it mainly depends on mitochondria's aerobic respiration. Additionally, glial cells that support the central nervous system rely on oxidative phosphorylation carried out by the mitochondria to maintain ion and water homeostasis. Due to heavy dependance of brain cells on the mitochondria, various neurological disorders can occur secondary to mitochondrial dysfunction.

Acute ischemic stroke is one of the leading causes of disability and death worldwide. Studies have shown that similar to other ischemic tissues, a common mechanism underlying the devastating effects of ischemia-reperfusion injury is oxidative stress, secondary to ROS (Figure 2) (Calio, Marinho et al. 2014, Anrather and Iadecola 2016). ROS activate astrocytes in response to ischemia-reperfusion injury, resulting in hypertrophy or astrogliosis (Pekny, Pekna et al. 2016). This hypertrophic event leads to excessive secretion of an intermediate filament cytoskeletal protein, glial fibrillary acidic protein (GFAP) (Pekny, Pekna et al. 2016), in which its increased expression inhibits regeneration in response to neural injury leading to scar formation (Sofroniew 2009). After the initial damage due to ischemia-reperfusion injury, mitochondrial dysfunction is a key player in triggering constant cellular energy disruption, oxidative stress, and neuronal loss (Anrather and Iadecola 2016). Currently, there are limited therapeutic options to mitigate acute ischemic stroke, leaving mitochondrial transplantation a potential therapeutic opportunity. Several animal studies have shown feasibility of mitochondrial transplantation to mitigate acute ischemic stroke. In a rat model, Zhang et al. (Zhang, Ma et al. 2019) delivered isogenic mitochondria harvested from the pectoralis major muscle through direct injection in the lateral ventricle following a 90-minute episode of ischemia in the middle cerebral artery (MCA) (Figure 3). They showed that following a 4-week recovery period, compared to a control group, rats receiving mitochondria exhibited reduced cellular oxidative stress and apoptosis, attenuated reactive astrogliosis, and enhanced neurogenesis. Furthermore, they observed reduced brain infarct size and reversal of some neurological deficits in the mitochondrial treatment group (Zhang, Ma et al. 2019).

Bejarpasi et al. (Pourmohammadi-Bejarpasi, Roushandeh et al. 2020) leverage human umbilical cord derived mesenchymal stem cells (MSCs) as a source of mitochondria for transplantation following ischemic stroke. After MCA occlusion in rat brains, these xenogeneic mitochondria were introduced via intracerebroventricular injection. They observed reduced apoptosis, decreased astrogliosis, reduced infarct size, and improved motor function, which delineates the therapeutic potential of xenogeneic mitochondria in alleviating the consequences of acute ischemic stroke.

Nakamura et al. (Nakamura, Lo et al. 2020) used cryopreserved mouse placenta as a source for transplantation of allogenic mitochondria and observed that 87% of placental mitochondria were viable for transplantation suggesting that the placenta may be a viable source for donor mitochondria.

These studies revealed that mitochondrial transplantation in the infarcted brain tissues is feasible through injection in the lateral ventricles or through an intravenous injection. Moreover, it is plausible that mitochondrial transplantation contributes to a neuroprotective effect after ischemia-reperfusion injury through reducing oxidative stress and apoptosis (Zhang, Ma et al. 2019, Nakamura, Lo et al. 2020, Pourmohammadi-Bejarpasi, Roushandeh et al. 2020), which resulted in the reversal of some neurological deficits and restored motor function and coordination (Zhang, Ma et al. 2019, Pourmohammadi-Bejarpasi, Roushandeh et al. 2020). Collectively, these studies delineate the potential of mitochondrial transplantation in reducing the morbidity and mortality associated with acute ischemic strokes.

Like the brain, spinal cord also heavily depends on mitochondria to meet its high energy demand. The spinal cord is susceptible to damage through ischemia reperfusion injury. Gollihue et al. (Gollihue, Patel et al. 2017, Gollihue, Patel et al.

2018) found that mitochondria can be injected into the spinal cord and can be taken up by the spinal cord neurons. They showed that direct injection of allogenic mitochondria either from cell culture or from skeletal muscle contributes to the maintenance of normal bioenergetics in a contusion spinal cord injury in rats (Gollihue, Patel et al. 2018). Despite the maintenance of normal bioenergetics, long-term functional protection of neuroactivity was not seen in those receiving mitochondria. Both studies required direct injection after laminectomy, a rather invasive technique not likely to be used in most clinical situations due to complications associated with the surgical procedure.

Spinal cord ischemia can lead to postoperative paraplegia during major aortic surgery (Miller, Patel et al. 2021). Fang et al. (Fang, Roan et al. 2021) showed that after spinal cord ischemia, allogenic mitochondria harvested from skeletal muscle delivered via the internal jugular vein improved lower-limb locomotor function of rats that were subjected to spinal cord ischemia up to seven days after surgery. Mitochondria were introduced before reperfusion occurred, ultimately resulting in attenuated tissue levels of interleukin-6, tumor necrosis factor-alpha, and caspase-3 in the mitochondrial treatment group. Ultimately, they observed that transplantation of allogenic mitochondria during the early stage of spinal cord ischemia reduces apoptosis, inflammation, and improves locomotor function (Figure 3) (Fang, Roan et al. 2021). Although introducing mitochondria prior to reperfusion seemed promising in reducing immune markers, the feasibility of this approach in clinical settings is perhaps only limited to a preventative measure in major vascular surgeries. Further studies are needed to investigate the use of mitochondria after ischemia-reperfusion injury, and to assess their long-term efficacy in maintaining normal motor function.

In addition to the classical manifestation of injury, either through ischemia or trauma, the CNS is also susceptible to cognitive and psychiatric disorders through currently unknown mechanisms. Nitzan et al. (Nitzan, Benhamron et al. 2019) tested the hypothesis that mitochondrial transplantation may ameliorate cognitive defects associated with Alzheimer's disease. In their study, a mouse model of Alzheimer's disease (amyloid-beta intracerebroventricularly injected) was treated intravenously with freshly isolated human mitochondria. Two weeks later, mice treated with mitochondria showed significantly better cognitive performance compared to their control counterparts, advancing towards the cognitive level of non-Alzheimer's mice (Figure 3). In addition to Alzheimer's disease, diabetes can also be a source of cognitive impairment. Ma et al. (Ma, Jiang et al. 2020) showed that platelet-derived mitochondria can be internalized into the hippocampal neurons within 24 hours of intracerebroventricular injection. A month after injection, diabetes-associated cognitive impairment was improved in mice treated with mitochondria (Ma, Jiang et al. 2020). Although the concept of treating cognitive disorders with mitochondria is still in its infancy, these studies exhibit exciting potential for the treatment of devastating cognitive diseases such as Alzheimer's.

Application of mitochondrial transplantation in cardiac ischemia-reperfusion injury

Much of the research on mitochondrial transplantation in the cardiac field has been focused on ischemia-reperfusion injury, as this is the most clinically relevant model of cardiac injury. Cardiomyocytes' contractile force demands high energy primarily in the form of ATP supplemented by the mitochondria. Calcium level, a key player in maintaining adequate electrical activity, is maintained at appropriate levels through calcium homeostasis driven by the mitochondria (Finkel, Menazza et al. 2015). In

fact, mitochondria are so crucial for the normal functioning of cardiomyocytes that they account for up to one third of the total cell volume (David, Bozner et al. 1981).

Given the vital role mitochondria play in the normal physiology of cardiomyocytes and during the pathology of ischemia-reperfusion injury, mitochondrial transplantation holds the potential to be a new therapeutic option. Masuzawa et al. (Masuzawa, Black et al. 2013) and Kaza et al. (Kaza, Wamala et al. 2017) observed that direct injection of mitochondria into the heart's infarcted area, in rat and swine models, leads to a decrease in inflammatory markers, e.g., high-sensitivity C-protein and IL-6, in the area at risk, and the infarct size significantly reduces compared to the controls. These studies suggest that mitochondria, delivered via direct injection, were received by the affected host cardiomyocytes, which may lead to better post-infarction recovery. Accordingly, direct mitochondrial injection may be clinically applicable in subjects undergoing post-infarct surgical repair.

In a typical clinical presentation of ischemia reperfusion injury, patients will not seek treatment until hours after the event. While previous studies focus on immediate injection of mitochondria, Blitzer et al. (Blitzer, Guariento et al. 2020) demonstrate the efficacy of delayed transplantation of mitochondria for cardioprotection. In their *in vivo* study, the investigators subjected Yorkshire pigs to 30 minutes of ischemia, allowing for 2 hours of reperfusion before introducing either sham control or autologous mitochondria. The authors demonstrate that delayed transplantation still provides cardioprotection, as pigs receiving autologous mitochondria had a significantly lower infarct size ($P < 0.001$) and enhanced ejection fraction ($P = 0.02$) (Blitzer, Guariento et al. 2020). These results demonstrate the clinical potential delayed mitochondrial transplantation may have on ischemia-reperfusion injuries.

Ischemia after removal of an organ is a driving force in limiting the feasibility of organ transplantation (Oweira, Ramouz et al. 2022). Moskowitzova et al. (Moskowitzova, Shin et al. 2019) hypothesized that mitochondrial transplantation may help increase the time between chilling an excised organ and having blood flow restored (cold ischemia time). They injected mitochondria both pre and postoperatively into excised hearts and showed that mitochondrial transplantation significantly increases ejection fraction in rat hearts after 29 hours of cold ischemic time when compared to hearts which did not receive mitochondria. They ultimately suggested that mitochondrial transplantation may play a role in enhancing graft function in organ transplantation- although significant more research in the field is needed before such a notion can be considered in the clinical setting (Moskowitzova, Shin et al. 2019).

Guariento et al. (Guariento, Blitzer et al. 2020) cannulated 26 Yorkshire pigs with sham control buffer or mitochondria, either serially or with a single bolus. After injection, the hearts were subjected to regional ischemia for 30 minutes and then reperfused for 120 minutes. They observed that pigs treated with mitochondria, either serially or with a single bolus, showed a significant increase in their coronary blood flow, ejection fraction, and LV pressure. They also observed a decrease in infarct size in pigs that prophylactically received mitochondria. The study suggests that mitochondrial transplantation may provide prophylactic cardioprotection from ischemia reperfusion injury. There was no significant difference between the hearts that received a single bolus of mitochondria compared to those that received serial injections (Guariento, Blitzer et al. 2020).

While direct mitochondrial injection to the myocardium may be applicable in some surgical procedures, a more clinically relevant model would rely on a minimally

invasive technique. Cowan et al. (Cowan, Yao et al. 2016) have demonstrated the effectiveness of mitochondrial transplantation through an intracoronary model. They effectively deliver mitochondria through coronary vasculature and report a reduction in infarct size comparable to the reduction in infarct size seen through direct injection of mitochondria. Shin et al. (Shin, Saeed et al. 2019) expanded on this technique in which mitochondria were delivered through an intracoronary catheter. Mitochondria tagged using ^{72}F-rhodamine-6G were visualized using positron emission tomography (PET) scans, which revealed a global distribution and uptake of mitochondria. While enhancing regional and global function of the left ventricle, they reported that mitochondrial transplantation did not affect heart rate, mean arterial pressure, or cardiac rhythm nor led to an increase in any apoptotic or inflammatory markers (Shin, Saeed et al. 2019).

The first clinical study of mitochondrial transplantation in humans involved pediatric patients with ischemia-reperfusion injury who were on extracorporeal membrane oxygenation (ECMO) (Emani, Piekarski et al. 2017). Isogenic mitochondria from skeletal muscle were isolated, suspended in respiration buffer, and rapidly administered to the affected area of the myocardium through direct epicardial injection with a syringe. Following isogenic mitochondrial transplantation, four out of five patients were successfully removed from ECMO, although ultimately only three survived (Figure 4). The autopsy of patients who succumbed to their injuries indicated no difference in pre- and post-injection markers of systemic inflammatory syndrome, suggesting no inflammation or rejection due to mitochondrial treatment. Moreover, a retrospective study examining mitochondrial transplantation in pediatric patients found that mitochondrial transplantation may be useful in separating from ECMO (Guariento, Piekarski et al. 2021). In this study,14 patients

underwent revascularization alone and 10 underwent revascularization with subsequent mitochondrial transplantation (Guariento, Piekarski et al. 2021). While 80% of patients treated with mitochondria were removed from ECMO within a week, only 29% of patients who did not receive mitochondrial treatment become ECMO free (Figure 4). Additionally, ventricular strain was significantly lower in the mitochondrial treatment group.

Ultimately, the above studies show promise for mitochondrial transplantation as a therapy for ischemia-reperfusion injury. For a more in depth overview of research involving mitochondrial transplantation in cardiomyocytes, refer to our previous article (Ali Pour, Hosseinian et al. 2021).

Application of mitochondrial transplantation in pulmonary vascular disorders

Mitochondria are the critical initiator of hypoxic pulmonary vasoconstriction (Paddenberg, Goldenberg et al. 2003, Wang, Zheng et al. 2007, Firth, Gordienko et al. 2009, Zhang, Zhou et al. 2012)—a crucial mechanism for maintaining an appropriate ratio of lung respiration and blood perfusion—which heavily contribute to the pathological development of pulmonary hypertension and edema. In contrast to systemic vessels in which hypoxia induces relaxation, in pulmonary smooth muscle endothelium, hypoxia leads to vasoconstriction (Leach, Sheehan et al. 2000, Michelakis, Hampl et al. 2002). It has already been established that mitochondria differ functionally and structurally in different cell types (Michelakis, Hampl et al. 2002, Firth, Gordienko et al. 2009, Zhou, Zhang et al. 2016). Zhou et al. (Zhou, Zhang et al. 2016) leveraged these structural and functional differences between pulmonary and systemic vasculature to reverse hypoxia induced pulmonary vasoconstriction *in vitro*. Their transplanted allogenic mitochondria from femoral

artery’s smooth muscle cells to pulmonary arterial smooth muscle cells reversed hypoxia-induced alterations in cell membrane potential, calcium ion homeostasis, and constriction of smooth muscle cells. Zhu et al. (Zhu, Zhang et al. 2016) expanded this research to investigate whether these effects could be seen *in vivo* in rats. They observed that mitochondrial transplantation via intravenous administration was feasible. The uptake of allogenic mitochondria attenuated acute hypoxic pulmonary vasoconstriction, reduced chronic hypoxia-induced pulmonary vascular remodeling, and mitigated the established pulmonary hypertension in rats exposed to chronic hypoxia.

Hsu et al. (Hsu, Roan et al. 2020) successfully induced pulmonary hypertension and left ventricular dysfunction through use of a rat model with left-to-right shunt aortocaval fistula and studied effects of mitochondrial transplantation after three weeks of recovery. Rats were treated with either placebo or allogenic mitochondria delivered via intravenous injection, harvested from the soleus muscle weekly, for three consecutive weeks. The ease of access to the soleus muscle provided adequate mitochondria delivered in a minimally invasive manner through intravenous injection. Mitochondrial transplantation significantly increased lung tissue ATP concentrations and improved right ventricular performance, even resulting in an increase in ventricular diameter (Hsu, Roan et al. 2020). Right ventricular mass and wall thickness were also restored in the group that received mitochondria. This study may help with establishing mitochondrial transplantation as a therapy for pulmonary hypertension secondary to structural heart defects (Lteif, Ataya et al. 2021). It can be inferred from this work that mitochondrial transplantation not only reverses pulmonary hypertension but may also help restore ventricular function induced by a left-to-right shunt.

Like the brain and heart, lungs are also prone to ischemic damage. Acute lung injury secondary to ischemia-reperfusion injury can occur due to a variety of surgical procedures and has been shown to have profound effects on lung viability, increasing morbidity and mortality in both adult and pediatric patients (Bernard, Artigas et al. 1994, Asimakopoulos, Smith et al. 1999, Edens, Chung et al. 2010, Huffmyer and Groves 2015, Naveed, Azam et al. 2017). Moskowitzova et al. (Moskowitzova, Orfany et al. 2020) showed the efficacy of mitochondrial transplantation in acute lung injury. In their experiment, transient ischemia was induced by applying a microvascular clamp on the left hilum for two hours. Upon reperfusion, mice received either a sham control or mitochondrial treatment through vascular delivery or by aerosol delivery via the trachea. This study revealed that mice receiving mitochondria had an increase in dynamic compliance and inspiratory capacity, resistance of the respiratory system, tissue damping, and elastance. Of particular interest in this study is the use of nebulized mitochondria—a novel non-invasive strategy for effectively delivering mitochondria to damaged lung tissue (Moskowitzova, Orfany et al. 2020).

Applications of Mitochondrial transplantation in cancer related issues

Mitochondria play a key role in regulation of cancer, specifically in breast and glial cell carcinoma (Zong, Rabinowitz et al. 2016). A common mechanism of action in anticancer drugs, such as doxorubicin, involves eliminating the cancer cells through mitochondrial dysfunction-induced apoptosis (Jeena, Kim et al. 2019). Alternatively, mitochondrial dysfunction in cancer cells has been shown to promote the progression of epithelial-to-mesenchymal transition, thereby promoting invasion, metastasis, and strong drug resistance (Zong, Rabinowitz et al. 2016). To make ATP, it is well known that many cancer cells switch from oxidative phosphorylation

to glycolysis, even in presence of abundant oxygen (Yu, Chen et al. 2017). Although appearing wasteful at first, as glycolysis generates a fraction of the ATP that oxidative phosphorylation produces, the rapidly dividing cells shunt glycolytic intermediates into nucleotide synthesis pathways (Warburg 1956). This phenomenon commonly known as the Warburg effect, allows for both adequate ATP generation and nucleotide synthesis, a requirement for the rapid and uncontrolled cell division (Warburg 1956, Vander Heiden, Cantley et al. 2009, Fogg, Lanning et al. 2011). As a result of this metabolic shift, lactic acid levels rise in the tumor microenvironment, contributing to cancer invasion, metastasis, radiation resistance, and drug resistance (Hirschhaeuser, Sattler et al. 2011, Choi, Collins et al. 2013). The molecular mechanism behind this metabolic shift is not well defined, but it is hypothesized that the replacement of abhorrent mitochondria with healthy mitochondria has potential for a new strategy in the fight against cancer (Seyfried 2015).

In one of the earliest studies of mitochondrial transplantation, Elliott et al. (Elliott, Jiang et al. 2012) demonstrated the effectiveness of mitochondrial transplantation in breast cancer cells. They showed that allogenic mitochondria inhibited proliferation and increased drug sensitivity in some cell lines. Although the mechanism of this phenomena was not fully clear, the group went on to test the hypothesis that healthy mitochondria replacing dysfunctional mitochondria promotes aerobic glycolysis. In their next set of experiments, Jiang et al. (Jiang, Elliott et al. 2015) demonstrated that allogenic healthy mitochondria suppress gene expression of glycolytic enzymes, lactate dehydrogenase A, and glucose transporter 1 and 3. Glycolysis assays revealed that allogenic mitochondria significantly suppress lactate production in culture media of the transfected cells. Collectively,

these studies show the potential of mitochondrial transplantation as a conjunctive therapy against epithelial breast cancer. Chang et al. (Chang, Chang et al. 2019) expanded on this theory by focusing on improving the effectiveness of mitochondrial transplantation through conjunction with a cell-penetrating peptide, pep-1. Their findings indicate that pep-1 increases the efficacy of mitochondrial transplantation in their coincubation studies (Chang, Chang et al. 2019).

Tumors reprogram pathways of nutrient acquisition and metabolism to meet the demands of their malignant cells. The metabolic shift from complete oxidation of glucose via oxidative phosphorylation to incomplete conversion of glucose to lactate via glycolysis is linked to activation of oncogenes, and to inactivation of tumor suppressor genes. The metabolic changes of tumor cause a scarcity of glucose in the tumor microenvironment where T cells directly compete for available glucose. As such T cells have a dynamic metabolic profile. Naïve T cells preferentially generate ATP through oxidative phosphorylation, while relying on glycolysis for activation, clonal expansion and effector function and need oxidative phosphorylation for survival and persistent antitumor response. Scharping et al. (Scharping, Menk et al. 2016) have shown that the immunosuppressive tumor microenvironment leads to dysfunctional T cells with mitochondrial defects, characterized by an overall loss of mitochondrial mass, which consequently leads to defect in oxygen consumption and bioenergetics. As such improving T cell mitochondrial function by increasing mitochondrial content via mitochondrial transplantation may restore the mitochondrial function in these cells and reduce the tumor burden by improving their antitumor response. This raises the question of whether it is possible to artificially reprogram the cancerous cells and/or immune cells to target and kill cancer cells and evade metastasis more effectively. We

propose two approaches (Kheradvar 2020, Kheradvar 2021): (1) Mitochondrial engineering as a positive regulator by “super-charging” T cells to more effectively attack cancerous cells. Based on the outcome of the study, this could potentially be a new addition to the current CAR T cell therapy, in which, the patient’s T cells are modified and a gene important to binding of T cells to cancerous cells is added to patient’s T cells. We have shown the feasibility of allogenic transplantation of mitochondria from human dermal fibroblasts NHDF-Neo to human Jurkats (immortalized T cells) visualized by confocal microscopy (Figure 5.A-B) The transplanted mitochondria were labeled with pHrodo Red SE, which is a pH sensitive dye that only fluoresces upon internalization by the cell. (2) Mitochondrial engineering as a negative regulator by transplanting diseased/dysfunctional mitochondria into cancer cells and investigating whether the post-transplanted cells are more prone to more attacks by the immune system and autophagy (Kheradvar 2020, Kheradvar 2021).

Application of Mitochondrial transplantation in other fields of Medicine

In addition to the above-mentioned applications, mitochondrial transplantation has been used to mitigate other disorders, although these studies are sparse. For example, mitochondrial transplantation has shown some efficacy in the treatment of acute kidney injury, an ischemic disease contributing to over 1.5 million deaths per year (Chen, Tang et al. 2019). Current treatment options are scarce and are limited to either organ replacement or hemodialysis, which are both kept as choices to treat the end-stage renal disease (Ibrahim, Ahmed et al. 2016, Lo, Sharma et al. 2016). Jabbari et al. have shown that isogenic transplantation of mitochondria via injection into the renal artery prevents renal tubular cell death, restores renal function, ameliorates kidney damage, and decreases ischemia/reperfusion-induced

apoptosis in rats (Jabbari, Roushandeh et al. 2020). Doulamis et al. (Doulamis, Guariento et al. 2020) showed the efficacy of isogenic mitochondrial transplantation via intra-arterial injection in Yorkshire pigs as transplantation led to renal protective effects as monitored by the decrease in serum creatinine and blood urea nitrogen. Ultimately, these studies provide encouraging preliminary support that mitochondrial transplantation may be used as a therapy against acute kidney injury, a condition with no true solution beyond management strategies.

Acute limb injury, a form of skeletal muscle ischemia-reperfusion injury, occurs in approximately 1 in 6000 and poses a major challenge for vascular surgeons (Sedghi, Collins et al. 2013, McNally and Univers 2018). Although treatable, all options are primarily invasive in nature with the 1-year mortality as high as 20% (Baril, Patel et al. 2013). Current non-invasive treatment strategies involve supporting vessel development and angiogenesis, which have shown limited efficacy given that adequate restoration of blood flow is not sufficient to rescue muscle viability (McNally and Univers 2018). As a type of ischemia-reperfusion injury, mitochondrial transplantation may hold promising therapeutic potential in preventing severe injury, improving morbidity and mortality. Orfany et al. (Orfany, Arriola et al. 2020) showed that mitochondrial transplantation ameliorates skeletal muscle injury and enhanced hindlimb function after acute limb injury in a mouse model. They showed that, through direct injection, mitochondrial transplantation significantly decreased infarct size and apoptosis. Mice receiving mitochondria also showed an increase in hindlimb function, as demonstrated in the increase of stance time.

Challenges and considerations in utilizing mitochondrial transplantation as a therapy

Although seemingly promising, there are still many considerations and reservations over mitochondrial transplantation. More specifically, there are some debates on whether mitochondria can survive external to their targeted cells and if they are able to mediate oxidative phosphorylation in the host cells. It is well established that calcium ions have detrimental effects on mitochondria, causing pore formation and subsequent destruction of the mitochondrial membranes (Gunter and Pfeiffer 1990). On that basis, Bertero et al. (Bertero, Maack et al. 2018) posed that mitochondria are unlikely to survive intracoronary delivery to the heart due to the high concentration of calcium ions in the blood. Bertero et al. (Bertero, O'Rourke et al. 2020) went on to report an experiment in which mitochondria suffer catastrophic damage in presence of 1 mM of Ca^{2+}, whereas others have reported mitochondria are able to survive up to 1.8 mM of Ca^{2+} (McCully, Emani et al. 2020). Considering that physiologic serum calcium level is well above 1 mM, Bertero et al. question whether or not mitochondria are truly able to survive transplantation mediated through intracoronary delivery, given that if the mitochondrial membranes are disrupted, pro-apoptotic and inflammatory mediators will be released into the blood, potentially mediating wide-spread inflammation and necrosis. McCully et al. (McCully, Emani et al. 2020) have gone on to refute these claims, citing several studies (King and Attardi 1988, Katrangi, D'Souza et al. 2007, Pacak, Preble et al. 2015, Kesner, Saada-Reich et al. 2016) which demonstrate the uptake and functional integration of exogenous mitochondria into recipient cells in media containing 1.8 mM of Ca^{2+}. They also cite several *in vivo* studies that show similar findings within animals with physiological calcium and sodium ions (Masuzawa, Black et al. 2013, Kaza, Wamala et al. 2017, Gollihue, Patel et al. 2018). In our opinion, a controlled study using cell culture media with different controlled ranges

of Ca^{2+} shall provide critical information about the potential limitations of mitochondrial transplantation in presence of different Ca^{2+} concentrations.

As well, there are other questions remain to be addressed on the efficacy of mitochondrial transplantation. For example, although there is evidence to suggest that mitochondria are rapidly taken up by the organ of interest, the number of functionally active mitochondria inside the cytosol is yet unknown. For the heart, some studies report as little as 2-7% of transplanted mitochondria are found inside of cardiomyocytes, and of those it is estimated that 80% are integrated into the mitochondrial network (Masuzawa, Black et al. 2013, Pacak, Preble et al. 2015). it is important to note that these studies justify the presence of transplanted mitochondria through retention of mitochondrial markers, but it is possible that the mitochondria that are up taken by the cells undergo membrane disruption or damage, while still retaining their markers, providing a falsely elevated concept of internalization. We are not aware of any studies that directly report the ratio of functionally active mitochondria or mitochondrial protein in the cytosol vs. the total number of transplanted mitochondria. Kaza et al. (Kaza, Wamala et al. 2017) report significant cardioprotection effect after mitochondrial transplantation, although they found as little as three mitochondria in a 5 µm thick histological sample of multiple cardiomyocytes. These findings raise the question whether the intact, whole mitochondria are truly responsible for the cardioprotective effects seen in the above studies, or if there is a secondary process at play. Bertero et al. suggest that the contents of the mitochondria, instead of the mitochondria themselves, mediate the clinical benefits noted by the numerous studies cited above (Bertero, O'Rourke et al. 2020). Indeed, more studies are required to address these questions in the field. It is our opinion that these questions, along with the remaining challenges to be

discussed, must be answered before mitochondrial therapy can be adopted in the clinical setting.

As the concept is still very young, mitochondrial transplantation must overcome many challenges to be considered as a therapeutic option in clinical settings. In our opinion, one question to be answered is which mitochondria can be used for each organ. Given that many cells with specialized functions (e.g., retina, cardiomyocytes, or neurons) may require particular levels of ATP generation and other specialized demands, it is important to understand which source of mitochondria can be used for transplantation in any particular cell. Once this question is answered the next challenge would be the availability of a particular type of mitochondria for a specific organ. Currently, studies on mitochondrial transplantation focus on isogenic, allogenic, and xenogeneic sources of mitochondria.

Even though studies have shown the feasibility of xenotransplantation for mitochondrial transplantation (Jiang, Elliott et al. 2015, Ali Pour, Kenney et al. 2020, Doulamis, Guariento et al. 2020, Popov 2021), the topic of xenotransplantation is still in its infancy and requires more investigation. Symbiosis theory of mitochondrial origin suggests that mitochondria in all eukaryotic lineages descend from a single common ancestor (Gray, Burger et al. 1999), and accordingly, retailoring the mitochondrial proteome has led to the gain and loss of protein components, resulting in diverse eukaryotic lineages that may prohibit closely related species to express or replicate mtDNA. (Kurland and Andersson 2000, Huynen, Duarte et al. 2013, Gray 2014). With mitochondrial transplantation being established, the safety and efficacy of xenotransplantation come into question. Several studies described here (Jiang, Elliott et al. 2015, Ali Pour, Kenney et al.

2020, Doulamis, Guariento et al. 2020, Popov 2021) utilize xenogeneic mitochondria as their source for mitochondria for transplantation, yielding seemingly promising results. Although encouraging, questions still remain on the long-term efficacy and safety of xenogeneic mitochondria as a source for transplantation. Long-term studies are needed to carefully assess whether xenogeneic mitochondria are capable of effectively replicating in the host.

While conventional methods of transplantation work in a controlled laboratory setting, in acute clinical settings, these methods may not be ideal, as mitochondria begin to rapidly degrade likely secondary to outer and inner membrane structural changes (McCully, Cowan et al. 2017, Bock and Tait 2020). If mitochondrial storage conditions can be improved so that mitochondria could be easily stored instead of immediately being harvested for each usage, the clinical applications would be vastly expanded. To address this limitation, Gnaiger et al. (Kuznetsov, Brandacher et al. 2000) tested and reported a buffer consisting of ATP, antioxidants, histidine, and colloid agents may offer greater preservation of mitochondria when stored under refrigerated conditions. Mitochondria isolated from rat hearts still show high respiratory capacity when stored on this buffer for over 24 hours, with an 80% retention of respiratory capacity (Kuznetsov, Brandacher et al. 2000). Moreover, they reported that addition of cytochrome C to the buffer maintains the respiratory capacity at nearly 100% over 24 hours. Although promising, this method fails to maintain respiratory capacity for more than 48 hours. Other buffers show similar efficacy over the course of 24 hours (Jassem, Armeni et al. 2006), but to date, no such cold storage mechanism is claimed to adequately preserve mitochondria for more than 48 hours.

Cryopreservation of mitochondria may address some of the fallings of cold storage of mitochondria. Greiff et al. (Greiff and Myers 1961) showed that, when stored at -65 ºC in a storage solution consisting of 10% dimethyl sulfoxide (DMSO), mitochondria maintained their full respiratory capacity after 18 days. The authors also claimed that under similar temperature conditions, mitochondria preserved in a storage solution consisting of glycerol maintained their full respiratory capacity after 15 days. Such observations, although initially promising, may not be a true solution for the storage of mitochondria, as Nakal et al. (Nukala, Singh et al. 2006) reported that mitochondria stored at -80 ºC in 10% DMSO show a slight reduction in respiratory capacity after just one week, citing 80% capacity as compared to their non-frozen counterparts. They also reported that membrane and cristae structure remain intact. Yamaguchi et al. (Yamaguchi, Andreyev et al. 2007) demonstrated that other buffer solutions, such as trehalose or a mannitol/sucrose solution result in reduction in respiratory capacity and membrane structure respectively. These studies underscore the need for adequate long-term storage of mitochondria.

Another major limitation that must be overcome before mitochondrial transplantation reaches its full clinical potential is the site-specific delivery. Much of the animal studies, and even human studies, have focused on direct injection of mitochondria into the target tissues. While practical in invasive surgical settings, this technique may not be ideal for most clinical scenarios where direct access would not be possible, such as treatment of ischemic strokes. Other practiced methods are intravenous injection of mitochondria to be delivered to the heart (Shin, Saeed et al. 2019) and lungs (Moskowitzova, Orfany et al. 2020). More recently, Moskowitzova et al. (Moskowitzova, Orfany et al. 2020) reproted mitochondrial transplantation through aerosal droplets in the lungs.

Conflicting evidence exists in regards to the safety of mitochondrial transplantation. Although several studies reported that allogenic transplantation of mitochondria are safe and do not mediate an autoimmune response (Masuzawa, Black et al. 2013, Kaza, Wamala et al. 2017, Shin, Saeed et al. 2019), other studies cited that mitochondrial components act as damage associated microbial patterns (DAMPs) (Collins, Hajizadeh et al. 2004, Struck, Uhlein et al. 2005, Iyer, Pulskens et al. 2009, Gollihue and Rabchevsky 2017, Yamada, Ito et al. 2020). These DAMPs are endogenous danger signals that are resleased from dead or dying cells that activate a potentially destructive immune response (Roh and Sohn 2018). Specifically, mitochondrial DNA, cytochome C, and the mitochondrial enzyme carbamoyl phosphate synthase have all been reported to act as DAMPs (Collins, Hajizadeh et al. 2004, Struck, Uhlein et al. 2005, Iyer, Pulskens et al. 2009, Gollihue and Rabchevsky 2017). Lin et al. (Lin, Xu et al. 2019) reported the ability of extracellular mitochondria to activate endothelial cells, resulting in an increase in cytokines and chemokines, crucial mediators of autoimmune activities. Pollara et al. (Pollara, Edwards et al. 2018) demonstrated that mitochondria-associated DAMPs are abundant in the systemic circulation of post mortem organ transplantation, likely causing a concomitant increase in proinflammatory cytokines and chemokines. Zhang et al. (Zhang, Raoof et al. 2010) showed that extracellular mitochondria can activate neutrophils with immunostimulatory effects. These reports pose a critical challenge to the safety of mitochdonrial transplantation through intravenous injection, which relies on extracellular mitochondria being systemically delivered to an organ. More studies are needed to understand the potential immune response to allogenic mitochodnria and innovate solutions to mitigate those.

Conclusions

The studies summarrized here collectively demostrate the potential of mitochondrial transplantation as a therapy in ameliorating mitochondrial function in cells and ultimately end-organ function. Although most studies focus on mitigating the outcomes of ischemic events, mitochondrial transplantation has shown efficacy to treat other disroders due to dynsfunctional mitochondria. Given that mitochondrial transplantation is in its infancy, more work needs to be done to address current callenges discussed above with respect to mitochondrial storage, route of admistration, and safety of mitochondrial transplantation. Looking to the future, mitochondrial transplantation holds the potential to serve as a therapy agaisnt many diseases spanning across multiple organ systems.

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Author Contributions

P.A. and A.K. conceived and designed research; P.A. performed experiments; P.A. analyzed data; P.A, interperted results of experiments; S.H. and P.A. prepared figures; S.H., P.A., and A.K. draftted manuscript; S.H., P.A., and A.K. edited and revised manuscript. S.H., P.A., and A.K. approved final version of manuscript.

Figure 1: Mitochondrial transplantation overview. Mitochondria can be easily and effectively harvested from the gastrocnemius mucsle or soleus muscle of a patient. After a brief digestion, filtration, and ultrifugation period the mitochondria are isolated and ready to be transferred. Mitochondria can either be transplantted through direct injection to the tissue of concern or through intravenous injection.

Figure 2: Mechanism of mitochondrial dysfunction in neural cells. After an ischemic insult, mitochondrial injury results in membrane damage, ultimately resulting in an increase in calcium and reactive oxygen species (ROS). Calcium overload results in ATP depletion whereas ROS in turn causes neurodegeneration through pro-inflammatory cyotokines.

Figure 3: Mitochondrial transplantation in neurological disorders. A) Mitochondrial transplantation have been shown to provide neuroprotective effects in icshemnic stroke. B) mitochondrial transplantation increase locomotor function and decrease inflammatory markers after spinal surgery. C) Mice subjected to Alzheimer's disease show increased cognitive performance after receiving intravenous mitochondria.

Figure 4: Mitochondrial transplantation in human subjects. To date, two such studies exist examining mitochondrial transplantation in humans. A) mitochondrial transplantation results in 80% of patients to come off of extra-corporeal membrane oxygenation (ECMO). B) patients receiving mitochondrial transplantation show greater recovery after icshemia-reperfusion injury.

Figure 5: Allogenic transplantation of mitochondria from human dermal fibroblasts (NHDF-Neo) into human Jurkat cells (immortalized T cells) visualized by confocal microscopy (Zeiss Laser Scanning Microscope 780, 100x, NA 1.4). **A.** Live cell imaging of the transplanted mitochondria which were labeled with pHrod Red, excited by 561 nm laser, and fluorescence was detected for 561-642 nm. **B.** Live cell imaging of control cells.

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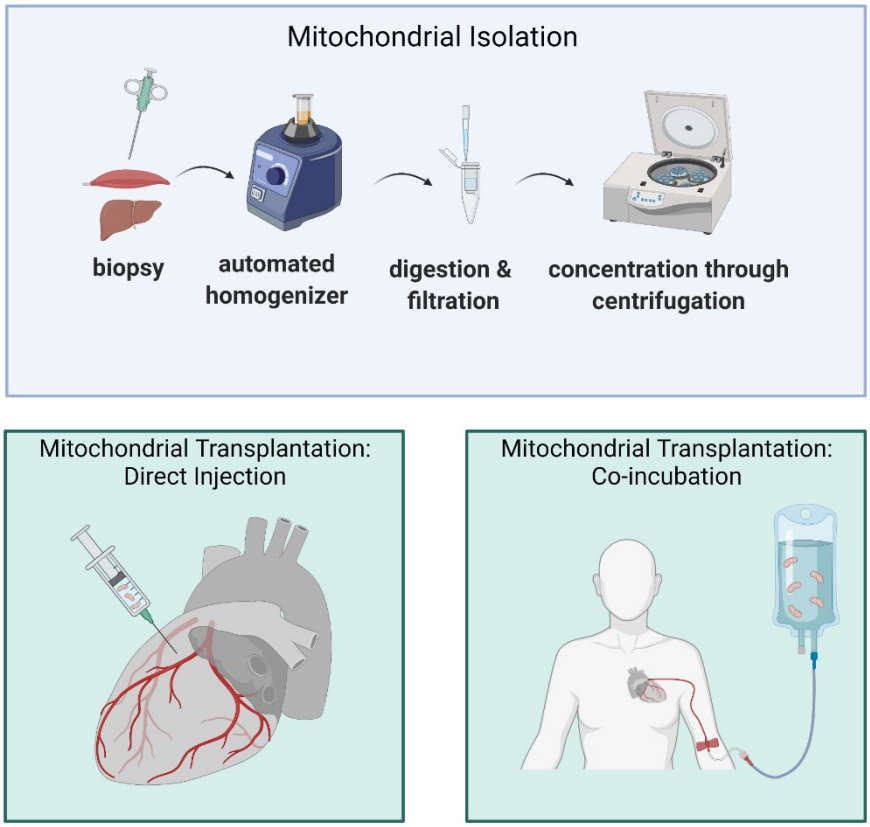

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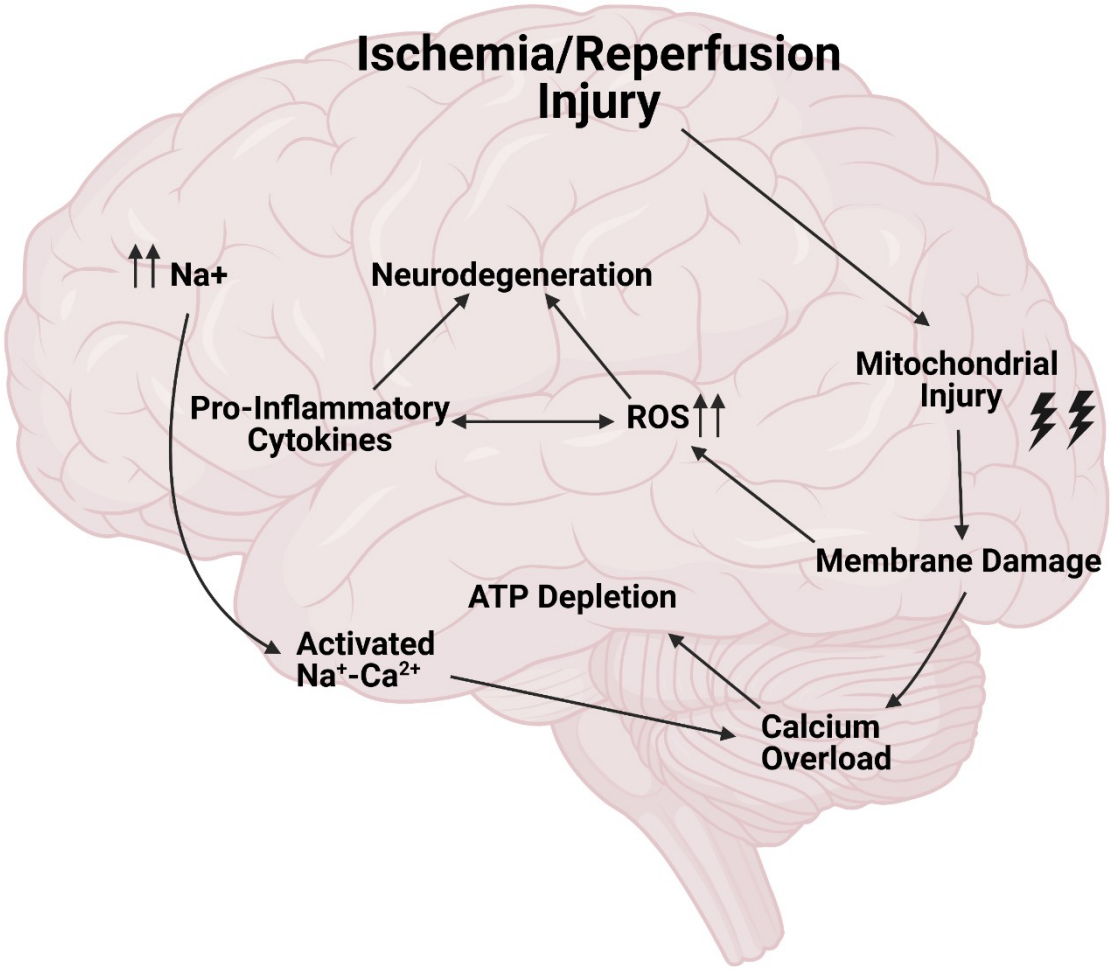

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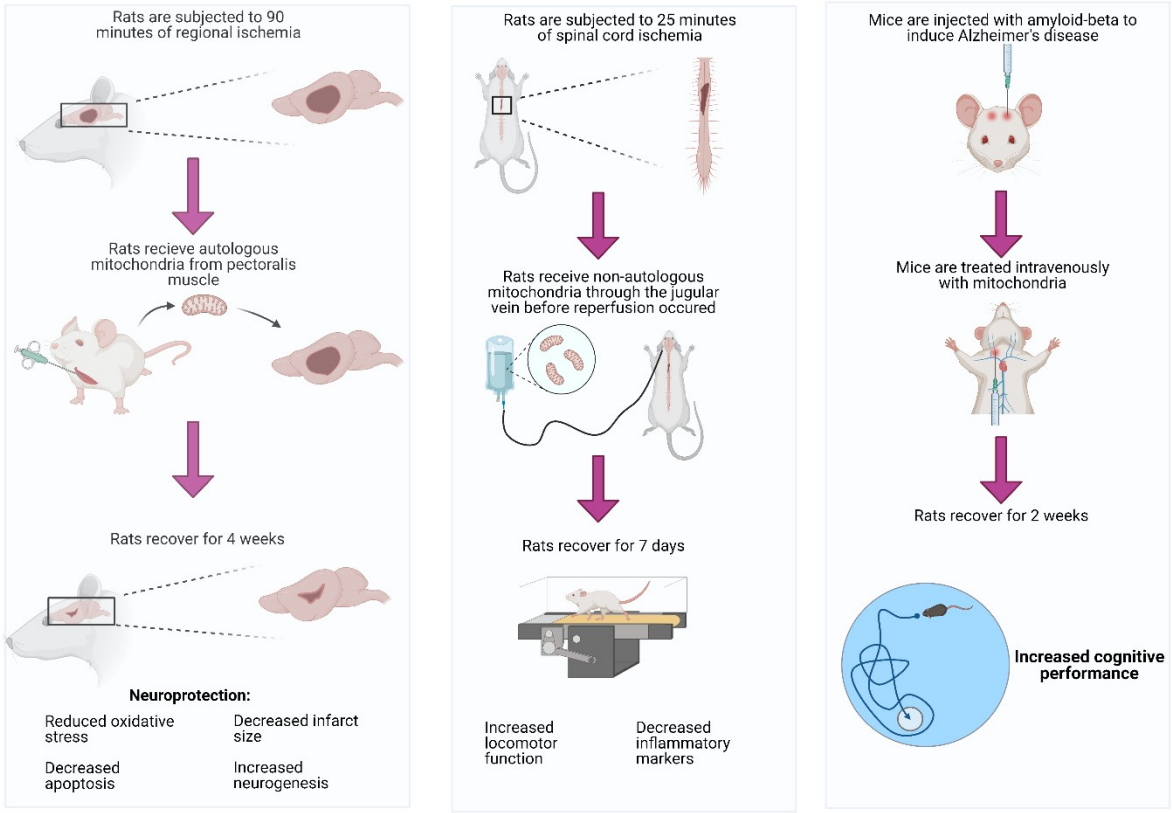

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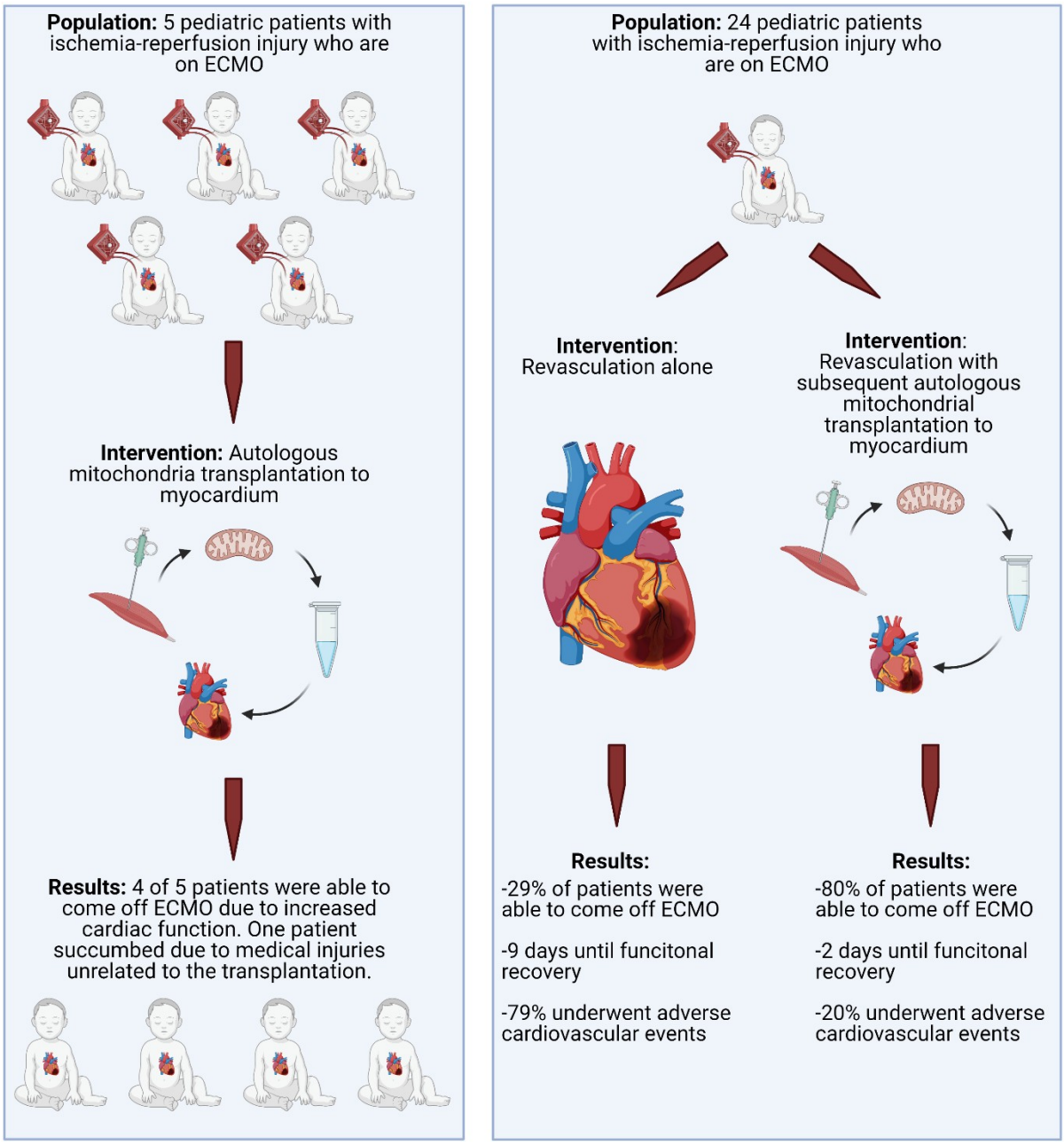

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