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Journal
American Journal of Physiology - Cell Physiology, 321(3)

ISSN
0363-6143

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Publication Date
2021-09-01

DOI
10.1152/ajpcell.00152.2021

Peer reviewed

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Keywords: Mitochondrial Transplantation, Mitochondrial Transfer, Mitochondrial Diseases, Mitochondrial Cardiomyopathy, Ischemia Reperfusion Injury, Bioenergetics.

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Abstract

Mitochondrial Transplantation is emerging as a novel cellular biotherapy to alleviate mitochondrial damage and dysfunction. Mitochondria play a crucial role in establishing cellular homeostasis and providing cell with the energy necessary to accomplish its function. Owing to its endosymbiotic origin, mitochondria share many features with their bacterial ancestors. Unlike the nuclear DNA, which is packaged into nucleosomes and protected from adverse environmental effects; mitochondrial DNA (mtDNA) are more prone to harsh environmental effects, in particular that of the reactive oxygen species (ROS). Mitochondrial damage and dysfunction are implicated in many diseases ranging from metabolic diseases to cardiovascular and neurodegenerative diseases, among others. While it was once thought that transplantation of mitochondria would not be possible due to its semi-autonomous nature and reliance on the nucleus, recent advances have shown that it is possible to transplant viable functional intact mitochondria from autologous, allogenic, and xenogeneic sources into different cell types. Moreover, current research suggests that the transplantation could positively modulate bioenergetics and improve disease outcome. Mitochondrial transplantation techniques and consequences of transplantation in cardiomyocytes are the theme of this review. We outline the different mitochondrial isolation and transfer techniques. Finally, we detail the consequences of mitochondrial transplantation in the cardiovascular system, more specifically in the context of cardiomyopathies and ischemia.

Providing adequate contractile force to propel blood throughout the body is the myocardium's primary function, which requires energy, primarily in the form of ATP supplemented by the mitochondria. Chemiosmotic hypothesis has long established that electron transport system (ETS) creates a proton gradient across the mitochondrion's inner membrane, which is used to drive ATP synthesis.(1, 2) Prior to this, most were convinced of the role of ETS in ATP synthesis with a provision that it is achieved based on a direct relationship through substrate-level phosphorylation.(3-5) Reactive oxygen species (ROS) at complexes I and III are the by-products of ETS, which can contribute to mitochondrial damage when in excess, and are part of the signaling sequence leading to apoptosis.(6) In cardiomyocytes, mitochondria are also responsible for regulation of calcium homeostasis to avoid irreversible ischemic heart damage due to cellular calcium overload.(7) Given the mitochondria's various functions in cardiomyocytes, together with cardiomyocytes' heavy energy demand, mitochondrial damage or dysfunction is associated with a variety of heart diseases. Mitochondrial dysfunction is commonly observed in cardiomyopathies, which can be due to ischemic damage or genetic mutations.(8)

Proper cardiac function depends on collective cellular bioenergetics and thus mitochondrial quality. Myocardium is the highest energy demanding tissue in the body,(9) with mitochondria accounting for up to one third of the total cell volume.(10) Cardiomyocytes require high energy to sustain their cellular function, and their demands increase during exercise or stress. The major source of energy for cardiomyocytes are carbohydrates in the fed state and fatty acids in the fasted state.(11) Fatty acid beta oxidation is less energy-efficient than glucose; previous studies have shown that supraphysiological free fatty acid (FFA) levels observed in heart failure or myocardial infarction, are due to high levels of norepinephrine.(11)

A cellular byproduct of oxidative phosphorylation is reactive oxygen species, or ROS. Normal levels of ROS are crucial for cellular communication, mediating different signaling pathways that

are ultimately responsible for maintaining homeostasis and adaptation to physiological stress.(12) Hence, it is the amount of ROS that determines whether a beneficial or harmful role is played. ROS play a role in oxidative modification of receptors, kinases, phosphatases, caspases, ion channels and transcription factors. In addition, they are implicated in signaling cascades that are important in cell fate, such as the protein kinase B (AKT), AMP-activate protein kinase (AMPK) and mitogen-activated protein kinase (MAPK) pathways.(13-15) Other recent studies have demonstrated the role of ROS signaling in a variety of other signaling pathways, activating p53, c-jun N-terminal kinase 1, and NF-kB.(16-18) Under physiological hypoxia, mitochondria release ROS, which subsequently activate hypoxia inducible factor 1, or HIF-1, a crucial mediator for metabolic adaptation under low oxygen circumstances.(19) ROS are also important in regulating synaptic plasticity-related signaling molecules, receptors, and channels, and critical for long-term potentiation, which is beyond the scope of the current review. Large amounts of reactive oxygen, nitrogen, and lipid species can lead to mtDNA damage and irreversible impairment of mitochondria, reduced ATP generation and eventually cell death. ROS-mediated activation of NHE-1 and CaMKII in cardiac hypertrophy and heart failure, respectively and ROS burst in ischemia-reperfusion injury are some examples of ROS involvement in the context of cardiovascular pathologies.(13)

Mitochondria also play important roles in calcium homeostasis. Calcium influx into the mitochondria is primarily regulated by mitochondria calcium uniporter (MCU). Ryanodine receptor 1 (RYR-1) and leucine zipper-EF-hand-containing transmembrane protein 1 (LETM1) are other mechanisms that have been proposed for calcium entry into mitochondria. A remaining question is whether LETM1 is in fact a Ca^{2+}–H^{+} exchanger or a K^{+}–H^{+} exchanger.(20) Calcium exits mitochondria through Na^{+}–Ca^{2+}–Li^{+} exchanger (NCLX).(20) The intracellular sodium level is maintained below the cytosolic sodium by sodium-proton exchangers to create

the electrochemical gradient necessary for the influx of sodium that is coupled to the efflux of calcium.(20)

Calcium plays a regulatory role in mitochondria and modulates the activity of mitochondrial dehydrogenases such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. Moreover, calcium leads to activation of complex V of the ETS, which converts the proton motive force to ATP. As such, an increase in work, leads to increased cytosolic and subsequently matrix calcium that in turn leads to increased ATP production.(20)While under increased work demand, increased mitochondrial calcium leads to increased ATP production; It is also calcium overload that triggers cellular necrosis under ischemia-reperfusion injury.(21) Furthermore, it has been shown that in heart failure an increase in cytosolic sodium is accompanied by increased matrix calcium, which leads to calcium efflux from mitochondria and reduced ATP production.(22)

Given that the dysfunctional mitochondria have a devastating role in the pathophysiology of variety of diseases, mitochondrial transplantation may offer exciting therapeutic applications. Although research on mitochondrial transplantation is currently dedicated predominantly to treatment of ischemic diseases in the context of cardiology and neurology, mitochondria play an important role in function of the kidney, gastrointestinal system, reproductive system, respiratory system, and hematological system, which is beyond the scope of this review. Here we summarize the current research surrounding mitochondrial transplantation in the context of therapies for cardiovascular diseases.

Mitochondrial transplantation includes the process of isolation (Figure 1), transfer, and internalization of exogeneous intact mitochondria into targets cell or tissue (Figure 2). Intracellular transplantation of these functional mitochondria has been proposed to repair damaged tissue in the brain, respiratory system, cardiovascular system, and renal system,(23-27) to name a few. It is hypothesized that owing to their endosymbiotic origin, the recipient cells

can uptake and integrate exogenous mitochondria and in doing so rescue cellular function and restore cellular homeostasis. Ali Pour et al.(28) and others have also shown the possibility of non-autologous/allogenic and even interspecies/xenogeneic mitochondrial transplantation.(28-31)

Mitochondrial isolation

Several techniques for isolation of mitochondria, based on differential centrifugation (DC),(32) density gradient high-speed ultracentrifugation (UC),(33) and magnetic cell separation (MACS)(34) have been practiced so far (Figure 1).

Original method of isolation relies on centrifugation at differential speeds to fractionate the harvesting cell's nuclear and cytoplasmic components and purify isolated mitochondria. This is achieved by initially centrifuging the cell suspension or tissue homogenate to pellet the nuclear fraction and cellular debris, followed by centrifugation at higher speeds to extract and purify mitochondria from the cytosol fraction. Another centrifugation-based technique uses a Ficoll sucrose density gradient and exceedingly high-speed centrifugation and buoyancy to isolate mitochondria.(34) However, with centrifugation techniques, it is difficult to achieve both high yield and purity. Since yield is high in DC, purity is lower compared to UC with roughly ~20% lysosomal and peroxisomal contaminants,(35) and although purity is higher in UC, yield is lower.

A more recent method known as fractionated mitochondrial magnetic separation (FMMS)(34) seems to lead to a high yield and high purity solution. Referred to as FMMS here, magnetic cell separation (MACS), which is a method typically used for separation of cell populations based on their surface antigens, has also been used in the context of mitochondrial isolation.(34) In FMMS, mitochondrion is first labeled with anti-TOM22, which is a magnetically tagged antibody against the translocase of the outer mitochondrial membrane 22. After this co-incubation, the labeled lysate is passed through a column within a magnetic field. Subsequently, the

magnetically tagged mitochondria will be trapped in the column and eluted, which leads to a higher purity isolate compared to the centrifugation techniques.(34)

Another method proposed by McCully et al.(27) relies on collection of autologous tissue samples massing below 0.1 gram from a subject through biopsy, homogenization of extracted tissues via an automated homogenizer, followed by brief digestion and filtration to break down cellular membranes and free organelles. This method may better meet the needs of clinical settings where an interventional time below 60 minutes is desired. Lastly, centrifugation is used to concentrate mitochondria, yielding approximately 1 x 10^9 mitochondria from a 0.1 g mass.(27) After the isolation, regardless of the used techniques, mitochondria can be immediately used for transfer and internalization (Figure 1).

Mitochondrial transfer and internalization

Mitochondrial transfer or internalization as a natural process has been observed in living cells, where mitochondria from one cell can be passed on to another via tunneling nanotubes, usually under conditions of cellular distress.(31, 36-39) However, in biomedical literature, mitochondrial transfer has been used interchangeably with transplantation, for artificial internalization of exogenous mitochondria. Here, we refer to exogenous delivery of mitochondria as transplantation.(40-43) Mitochondrial transplantation has been attempted through direct injection of isolated mitochondria to the recipient tissue,(31, 44-46) co-incubation,(28, 29, 31, 47-51) centrifugation,(52) magnetomitotransfer (use of electromagnetic forces),(40) and photothermal nanoblade (Figure 2).(41)

Direct injection has been used in the *in vitro* settings, animal models, and in even clinical settings, where the isolated mitochondria are directly injected into the recipient cells (through microinjection) or tissue (via needles).(44-46)

Co-incubation, as the name indicates, involves the incubation of the isolated mitochondria and recipient cells in the vicinity of one another to allow the recipient cells uptake mitochondria,

158 perhaps similar to the way in which ancestral mitochondrion, once a bacterium, was taken up by
159 the ancestral eukaryotic cell (Figure 3 and Figure 4).

160 Centrifugation at 1,500 x *g* for 5 minutes has been shown to enhance the delivery of exogenous
161 mitochondria when compared to the simple co-incubation method. In this technique, once the
162 isolated mitochondria are mixed with the recipient cells, the co-incubated sample is briefly
163 centrifuged to enhance and accelerate the mitochondrial uptake (Figure 3).(52)

164 *Magnetomitotransfer* is accomplished first by labeling mitochondria with anti-TOM22, and then
165 exposing these magnetically-labeled mitochondria to a magnetic plate. Magnetomitotransfer has
166 a reported efficiency of 78-92% compared to co-incubation's efficiency of 17%.(40) Additionally,
167 this technique can increase mitochondrial internalization evidenced by a higher ratio of cells and
168 higher density of transferred mitochondria within the first day of the recipient cell culture.(40)

169 Finally, a *photothermal nanoblade* procedure has been reported to be used to transfer isolated
170 mitochondria into somatic mammalian cells.(41) In their proof-of-principle study, Wu et al.(41)
171 showed that their nanoblade apparatus was successful in transferring isolated mitochondria,
172 leading to restoration of metabolic gene expression, global energetic, and metabolite profiles.
173 Although the application of such a mechanism may be limited, their protocol using a precise
174 apparatus has the potential to protect against the transfer of other unwanted cytosolic
175 biomolecules such as microRNAs and abhorrent signaling molecules that can impact cellular
176 biologic functions.(41)

177 Nevertheless, mitochondrial transplantation studies are still in their infancy and more needs to
178 be done to assess its efficiency and efficacy. Evaluating the efficiency of mitochondrial
179 transplantation is challenging particularly considering discrimination and proper quantification of
180 native and transplanted mitochondrial population. Although these organelles seem to be
181 independent, they are in fact interconnected through a dynamic reticulated network, allowing to
182 share their contents.(31, 53) Mitochondria can assume different sizes and shapes through

fission and fusion processes, and lack of fluorescently stabilized cell lines that allow continuous monitoring of native and transplanted mitochondria, make the comparison of native and transplanted population (and hence determining transplantation efficiency) challenging. Given the recent availability of HiPSCs with stabilized fluorescently tagged mitochondria (mECFP, and mTagRFP), in our opinion, further quantitative studies with more consistent results can be performed in the near future.

Maeda et al.(54) attempted to increase mitochondrial transplantation efficiency using TransActivator of Transcription dextran (TAT-dextran) complexes by enhancing cellular uptake of exogenous mitochondria. They suggest that TAT-dextran complexes enhance cellular uptake of exogenous mitochondria and can improve the protective effect of mitochondrial replenishment in rat cardiomyocytes against oxidative stress. When compared to co-incubation, TAT-dextran transferred mitochondria showed a lower expression of apoptosis-promoting gene *Bax* to apoptosis-suppressing gene *Bcl-2*, indicating a more effective anti-apoptotic shift. TAT-dextran modification also increased internalization of exogenous mitochondria as confirmed by flow cytometric analysis (183 ± 18.4%, $P < 0.01$ versus co-incubation group).(54)

Actin-dependent endocytosis and macropinocytosis have been speculated as possible mechanisms of internalization of the transplanted mitochondria into a recipient cell. More studies are necessary to establish the mechanism or mechanisms of mitochondrial internalization. In our opinion, perhaps more than one mitochondrial internalization mechanism could be involved in internalization of exogenous mitochondria by recipient cells with potential dependency on the recipient cell/tissue type and mode of mitochondrial delivery. Pacak et al.,(26) investigated blocking caveolae-mediated, clathrin-mediated endocytosis, actin-dependent endocytosis, macropinocytosis, and tunneling nanotubes using pharmacological blockers of endocytosis pathways, i.e., Methyl-b-cyclodextrin (MbCD), Cytochalasin D (CytoD), 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), and nocodazole (Noco), respectively. Prior to

mitochondrial transplantation, the cells were incubated with one of the above-mentioned pharmacological blockers. Mitochondrial internalization was only significantly decreased in the presence of CytoD, which suggests the involvement of actin-dependent endocytosis pathway.(26) A more recent work by the same group, Cowan et al.(55) used 3-D super-resolution microscopy and transmission electron microscopy to visualize exogenous human mitochondria post-transplantation and looked more closely at the dynamic of intracellular trafficking of mitochondria. With respect to mitochondrial internalization through endocytosis, mitochondria are taken by the cells through endocytic vesicles formed by the invagination of the cellular membrane during the cellular uptake process. These vesicles are then fused with early endosomes as a major sorting comportment. Once the endosome reaches the cells, it should ideally release its contents (i.e., mitochondria). However, endosomal escape can be a limiting step if the mitochondria get trapped inside the endosomes. If mitochondria are destined to be degraded, they will be transported to late endosomes, which together with Golgi's lysosomal comportments will form the endolysosomes that can provide the proper environmental condition for degradation and recycling of material. Yet, some cargo might be able to escape the late endosomes. In their studies, Cowan et al. (55) labeled early and late endosomes with early endosome antigen 1 (EEA1) and late endocytic marker Rab7, respectively. They assumed some of the transplanted mitochondria were degraded based on their fragmented appearance in some lysosomes. However, the majority of internalized GFP-labeled mitochondria successively progressed through the endolysosomal system, successfully escaped the endosomal and lysosomal compartments, and effectively fused with the endogenous mitochondria based on their association with the cardiomyocyte mitochondrial network.(55)

Although studies by Pacak et al.(26) and Cowan et al.(55) suggest actin-dependent endocytosis as the mechanism for mitochondrial internalization in cardiomyocytes, previous studies by Kitani et al.(56) and Kesner et al.(57) suggest macro-pinocytosis involvement in the context of

internalization of mitochondria in human uterine endometrial cancer cells. In studies by Kesner et al.,(57) using transmission electron microscopy, they observed mitochondria were engulfed by the recipient cells through cellular extensions and cell surface ruffling. This led them to hypothesize macropinocytosis involvement, which was confirmed when mitochondrial uptake was blocked upon use of micropinocytosis inhibitors except the clathrin-mediated endocytosis inhibitors.

With respect to this disagreement on the underlying mechanism of mitochondrial internalization, Patel et al.(58) argue that with the exception of the work by Kesner et al., current knowledge may not suggest a conclusive and reliable decision since the studies only rely on use of endocytotic inhibitors, which can inhibit multiple endocytic pathways.

Consequences of Mitochondrial Transplantation in the Cardiovascular System

Mitochondrial transplantation improves outcomes in ischemia-reperfusion injury

Ischemia-reperfusion injury in the context of ischemic heart disease is defined as the paradoxical exacerbation of cellular dysfunction and death following restoration of blood flow to previously hypoxic tissues.(59) Ischemic damage to mitochondria leads to their malfunction. Since mitochondria's DNA encodes most of their components, prolonged period of ischemia can permanently damage the ability of cardiomyocytes' mitochondria to generate ATP.(60, 61)

It is currently believed that irreversible ischemic damage may be due to: (1) loss of ATP, (60, 61) (2) supraphysiological free fatty acids (FFA) levels,(11) (3) formation of excess reactive oxygen species (ROS),(62-64) (4) cellular calcium overload,(65-68) and finally (5) sodium pump inhibition (Figure 5).(69, 70)

In unstressed healthy individuals, although ROS are continually generated during cellular metabolism, they are effectively removed by cellular antioxidant systems. An example of this antioxidant system is superoxide dismutase that removes superoxide radicals produced during

cellular metabolism.(71) Under normal circumstances, these antioxidant systems are efficient at maintaining ROS at a healthy level. However, it is during ischemia when ROS production exceeds the “buffering” capacity of the antioxidant system, leading to disruption of calcium homeostasis, mitochondrial and cellular DNA damage, and mitochondrial lipid peroxidation.(72, 73) Ultimately, this leads to cell death by apoptosis through the intrinsic apoptosis pathway.(62-64)

In the classical manifestation of ischemia, occlusions in the arterial supply leads to the hypoxic effects described earlier. Restoration of blood flow, or reperfusion, is frequently found to exacerbate tissue injury. In fact, reperfusion can lead to an additional 40% cardiac cell death.(74) This may be due to the altered metabolism of ischemia-damaged mitochondria, leading to excessive ROS production and disruption of the mitochondria membrane.(75) Reperfusion also elicits rapid alterations in ion flux, and this rapid change of pH may lead to enhanced cytotoxicity.(69, 70) To return to normal pH levels, the cell utilizes sodium-dependent pH regulators, such as the Na^+–H^+ exchanger. Once activated, hydrogen is pumped out of the cell, leading to intracellular sodium accumulation. High sodium levels activate Na^+–Ca^{2+} exchangers, leading to Ca^{2+} overload, which in turn causes ATP depletion and damage to mitochondria.(65-68)

Metabolic and ionic changes secondary to the lack of oxygen are major contributors to the mitochondrial damage in the myocardium during ischemia, which significantly affect cardiac function and cell survival during ischemia reperfusion injuries. Masuzawa et al.(44) hypothesized and tested that in ischemia, cell survival will improve by transplantation of autologously derived mitochondria, immediately prior to reperfusion. In their *in vivo* and *in vitro* studies in New Zealand White rabbits and Lewis rats, autologously derived mitochondria were injected into regional zones of ischemia, following 29 minutes of reperfusion injury, and the hearts were allowed to recover for 4 weeks. Their results showed that the transplanted

mitochondria are internalized by the tissue in 2 to 8 hours. Moreover, cardiac enzymes, creatine kinase MB and cardiac troponin-I, apoptosis, and infarct size significantly decreased post-transplantation in the reperfusion injury zone. Additionally, within 10 minutes after reperfusion, normal contraction was re-established in the transplant group, while in the control group hypokinesia in the reperfusion injury zone persisted until 4-week recovery timepoint (Figure 7).(44)

Cowan et al. (47) expanded on their previous work(44) and demonstrated the effectiveness of transplantation in an intracoronary delivery model. They effectively delivered mitochondria through coronary vasculature in Langendorff-perfused rabbit hearts that were subjected to 30 minutes of ischemia and then reperfused for 10 minutes. They concluded that mitochondrial transplantation through coronary arteries was practicable and occurred within minutes. Mitochondria delivered via coronary arteries spread throughout the cardiac tissue, whereas those locally injected were predominantly localized to the interstitium of the injection site. Vascular delivery of mitochondria reduced infarct size similar to direct injection of mitochondria, which indicates a clinically relevant method, since direct injection may not be feasible in all interventional settings.(47)

Previous work has demonstrated that mitochondria from diabetic rats are dysfunctional, having reduced ATP production and respiratory capacity.(76) Doulamis et al.(29) investigated whether the source of allogenic mitochondria played a part in improved clinical outcomes, by comparing mitochondria isolated from either non-diabetic or diabetic rats. Their results demonstrate that mitochondrial transplantation enhances post-ischemic myocardial function and reduces cardiomyocyte injury in diabetic rat hearts regardless of the source of mitochondria (Figure 7). Both groups of diabetic rats receiving mitochondria exhibited an increase in left ventricular (LV) pressure after transplantation. In diabetic rats that received allogenic mitochondria, mean LV pressure was 92.8 ± 5.2 mmHg, 110.7 ± 2.4 mmHg, and 44.3 ± 5.9 mmHg in rats that received

mitochondria from diabetic, non-diabetic, and healthy control donors, respectively. Similar improvements were observed for infarct size, with sizes of 11.8 ± 0.7, 9.9 ± 0.5%, and 52.0 ± 1.4%, in rats that received mitochondria from diabetic, non-diabetic, and healthy control donors, respectively.

There was no significant difference in cardiac function or myocellular viability between mitochondria-receiving groups, suggesting that mitochondrial transplantation has great potential to reduce morbidity and mortality in diabetic patients subjected to myocardial ischemia-reperfusion injury.(29)

McCully's group continued their mitochondrial transplantation studies in swine models; Kaza et al.(45) performed mini-thoracotomies on Yorkshire pigs with pectoralis major dissected and removed to be used for isolation of autologous mitochondria. After 24 minutes of ischemia-reperfusion injury, the hearts received multiple injections of either a sham control or vehicle containing mitochondria into the area at risk. Levels of creatine kinase-MB and troponin I were significantly decreased in the treatment group compared to the sham group, which persisted for at least 4 weeks post transplantation. Immune, inflammatory, and cytokine markers showed no difference among the groups. While there was no significant difference in the area at risk between the sham control and vehicle containing mitochondria ($P = 0.48$), the infarct size was significantly reduced ($P = 0.004$) in the group receiving mitochondria. Multiple doses of mitochondria were tested, with no significant difference in decreasing the infarct size despite the number of mitochondria injected.(45)

The swine model was also used to test the prophylactic effect of mitochondrial transplantation for the hearts exposed to ischemia reperfusion injury (Figure 7). Guariento et al.(50) cannulated 26 Yorkshire pigs with sham control buffer or mitochondria, either serially, having 10 injections over 60 minutes, or with a single bolus. The hearts were subjected to regional ischemia, 15 minutes after injection, for 30 minutes and were reperfused for 120 minutes. They found that

both groups of pigs receiving mitochondria had significantly increased coronary blood flow, ejection fraction, and improved LV pressure. Like the previous findings, there was no difference in the size of the area at risk, but pigs receiving mitochondria did have a significantly smaller infarct size, suggesting mitochondrial transplantation provides prophylactic cardioprotection from ischemic reperfusion injury. Most notably, there was no significant difference between those who received a single bolus of mitochondria versus those who received serial injections, suggesting no advantages for the latter.(50)

Although previous studies focus on immediate injection or cannulation of mitochondria, in a typical manifestation of ischemia reperfusion injury, patients will typically not seek treatment in the minutes or hours following an ischemic event. Blitzer et al.(49) investigated the efficacy of delayed transplantation of mitochondria for cardioprotection in a swine model by subjecting Yorkshire pigs to 30 minutes of ischemia, allowing for 120 minutes of reperfusion, and then introducing either sham control or autologous mitochondria to the coronary ostium. The authors suggest that delayed transplantation still provides cardioprotection, as pigs that received mitochondria after the delay had a significantly enhanced ejection fraction ($P = 0.02$) and significantly lower infarct size ($P < 0.001$), which further underscores the clinical application of mitochondrial transplantation.(49)

Mitochondrial transplantation may also play a role in improving the efficacy of organ transplantation. Moskowitzova et al.(51) hypothesized that mitochondrial transplantation may increase cold ischemia time in heart transplants. Cold ischemia time is defined as the time between the chilling of a tissue after its blood supply has been cut off and the time it is warmed by having blood supply restored. Moskowitzova et al.(51) suggest that this type of ischemia-reperfusion injury also benefits from mitochondrial transplantation. Pre- and post-transplantation injections of either sham control (heparin) or vehicle containing mitochondria to transplanted rat hearts with a cold ischemic time of 29 hours, followed for the next 24-hour, suggest that beating

score, ejection fraction, and shortening fraction were significantly enhanced ($P < 0.05$). Neutrophil infiltration and necrosis were significantly decreased ($P < 0.05$), ultimately suggesting that their protocol can enhance graft function and reduce graft failure in transplant patients.(51)

In many countries donation after circulatory death (DCD) as a measure for organ transplantation describes regulations related to the retrieval of organs for the purpose of transplantation that follows death confirmed using circulatory criteria. Warm ischemia is a serious concern to this procedure as it affects the entire organ.(77) Guariento et al.(78) recently investigated whether mitochondrial transplantation can help address this concern by increasing cardiac efficiency. They had 29 Yorkshire pigs undergone circulatory death by cessation of mechanical ventilation. After 20 minutes of warm ischemia and 15 minutes of reperfusion via an ex-situ blood perfusion system, the hearts received either a sham control or vehicle containing autologous mitochondria. A third group received a second injection of mitochondria after two hours of *ex-situ* heart perfusion. The hearts which received autologous mitochondria showed a significantly increased left ventricular pressure, fractional shortening, and myocardial oxygen consumption. Infarct size was also significantly reduced in hearts receiving mitochondria when compared to the control group with sham treatment.(78)

The question of efficacy and safety of mitochondrial transplantation remains to be addressed. Accordingly, Shin et al.(48) developed a novel method for delivering mitochondria through an intracoronary catheter. To investigate mitochondrial biodistribution, mitochondria were tagged using ^{72}F-rhodamine-6G and were visualized using positron emission tomography (PET) scan. Visualization revealed global distribution of the mitochondria. These studies also found that intracoronary injection of mitochondria at any tested concentration did not affect heart rate, mean arterial pressure, or cardiac rhythm, but enhanced regional and global LV function. Intracoronary injection increased coronary blood flow in a concentration-dependent scheme without causing any detectable lesions or blockages. The authors used antidiuretic hormone

(ADH) to induce myocardial stress and increase myocardial demand. Injection of ADH resulted in coronary vasoconstriction and decrease in coronary blood flow, which was rescued by intracoronary injection of mitochondria. Ultimately, this study reveals the safety and efficacy of mitochondrial transplantation via coronary catheters. This mechanism of bolus delivery makes mitochondrial transplantation more applicable for use in clinical settings.(48)

The first clinical trial of mitochondrial transplantation recruited pediatric patients with ischemia-reperfusion injury who were on extracorporeal membrane oxygenation (ECMO) support.(79) Autologous mitochondria from nonischemic skeletal muscle were isolated, suspended in 1 mL respiration buffer, and administered to the affected myocardium through direct epicardial injection with a tuberculin syringe, and monitored by epicardial echocardiography to assess hypokinesis. Following autologous mitochondrial transplantation, four in five patients were successfully removed from ECMO support, although ultimately only three survived. Additionally, the autopsy of patients who could not be removed from ECMO indicated no difference in pre- and post- injection markers of systemic inflammatory syndrome suggesting no inflammation or rejection due to mitochondrial treatment.(79)

Although this study was limited in its sample size to draw any statistically significant conclusion, it paved the way for their next pilot study examining autologous mitochondrial transplantation in pediatric patients.(46) In this retrospective study of patients requiring ECMO, 14 patients underwent revascularization alone and 10 underwent revascularization with subsequent mitochondrial transplantation. While 80% of the patients who received mitochondrial transplantation were removed from ECMO within 1 week, only 29% of the patients in the revascularization alone group became ECMO free ($P = 0.02$).(46) Ventricular strain was significantly lower in the mitochondrial recipient group. Median functional recovery time after revascularization was also significantly shorter with fewer adverse cardiovascular events.(46)

Ultimately, this study suggested that mitochondrial transplantation is associated with successful separation from ECMO and better cardiac function overall.(46)

Mitochondrial transplantation improves cellular Bioenergetics in healthy cardiomyocytes

One of the many aims of mitochondrial transplantation is to modulate cellular bioenergetics, and in turn function, in the recipient cells. Recent studies by Ali Pour et al.,(28) demonstrate short and long term (up to 28 days) post-transplantation bioenergetics in healthy cardiomyocytes in . They show that cellular bioenergetics *in vitro* is acutely improved post-transplantation; however, this enhancement is short-lived and the improved bioenergetic indices (basal respiration and ATP production) return to normal rate biological levels in long-term. This observation suggests that mitochondrial transplantation may boost healthy cellular energetics, which can be clinically useful in mitigating acute myocardial stress.(28) However, more research is needed to investigate mitochondria's potential and efficacy in treatment of more chronic conditions.

Mitochondrial Diseases and Dysfunction

Mitochondrial cardiomyopathy is characterized by abnormal myocardium secondary to genetic defects that leads to impairment or loss of the factors in the mitochondrial respiratory chain, in the absence of other cardiac diseases (Figure 6).(80) Cardiomyopathies are some of the most frequent manifestations of mitochondrial diseases, occurring in 20-40% of children with mitochondrial disease.(81, 82) Clinical manifestations of mitochondrial cardiomyopathies can vary in severity from asymptomatic to severe symptoms, most notably heart failure and arrhythmias. Mortality in children with mitochondrial diseases is higher in those with cardiomyopathy than in those without.(82)

It is speculated that the etiology of mitochondrial cardiomyopathy lies in the impairment or loss of the electron transport chain (ETC) complex subunits and CoQ_{10} synthesis.(80) Cardiomyopathy has been reported in patients with mutations in mitochondrial and nuclear

genes encoding complex I subunits, such as MTND1 and MTND5.(83) Mutations in genes encoding for complex II subunits, such as SDHA and SDHD, have also been reported in patients with cardiomyopathies.(84)

The prevalence of mitochondrial diseases is much higher than previously estimated. It is currently estimated that mitochondrial diseases occur in 1 in 4,000 individuals, which could still be an underestimate due to misdiagnosis.(85) Presently, no clinically proven therapy is available for mitochondrial diseases, which highlights the unmet clinical needs.

Potential therapies envisioned are broadly divided into gene-based and cellular therapies. Cell-based therapies are established on the transplantation or delivery of intact new organelles from healthy cells to diseased cells, which may potentially improve outcome for patients who suffer from the consequences of acquired or congenital mitochondrial defects merely due to mitochondrial mutations. This can be achieved through intercellular transfer of exogenous mitochondria, potentially from allogenic sources. Several studies have also shown the efficacy of intercellular transfer from stem cells to diseased cells(36, 86-89) and transfer of exogenous mitochondria via transplantation is currently being investigated.(31, 90)

Mitochondrial genome, similar to the nuclear genome, is constantly exposed to external and internal agents that can result in damage and mutation.(91) Mitochondrial diseases develop as a result of the clonal expansion of the mutated/damaged mtDNA [Figure 6]. Different tissues have different thresholds for mtDNA mutation load that can lead to cellular mitochondrial dysfunction, with the threshold being lower for cells that depend heavily on the energy supplied by mitochondria, such as cardiac cells.(92) Since mitochondria play a critical role in the cell, the maintenance of mtDNA integrity is vital to the well-being of the cell. Thus, mitochondria constantly undergo fission and fusion processes to maintain their fitness.(93)

mtDNA is packaged into DNA-protein assemblies called nucleoids.(94) Fusion as the name implies is the process where two mitochondria join and exchange their nucleoids.(95, 96).

Fission is the reverse process, in which there is an increase in number of mitochondria.(95, 96) Fusion is believed to have a protective role in pathologies caused by mtDNA mutations.(95) It has been shown that fusion allows for complementation effect where two nucleoids, each with different mutations in the same mitochondria, display the wild-type phenotype.(97, 98) However, when the mutation is severe, it would be best to isolate and remove the mutation through fission, in which the mutant nucleoids are segregated into a mitochondrion (or few mitochondria) that can be marked for degradation by the mitochondrial autophagy, commonly referred to as mitophagy.(96)

With the accumulation of deleterious mutation, nucleoid replication also increases by the retrograde signaling to produce more nucleoids so that the mitochondria would be able to meet the demand of the cell, which is another mechanism mitochondrion employs in maintaining its proper function and providing the cell with energy.(99)

Although mitochondria lack a true DNA repair mechanism, they use fission and fusion processes to maintain a healthy network of mitochondria and this characteristic is the reason why we believe mitochondrial transplantation may have the potential to serve as a cellular biotherapy in the context of mitochondrial diseases due to mtDNA mutations. If the transplanted mitochondria are adopted by the recipient cells for long-term, transplantation of healthy mitochondria can dilute the mutation load. Moreover, with an effective treatment plan (appropriate mitochondrial dose and frequency of treatment), which can be established using mathematical modeling of mitochondrial dynamics in conjunction with experimental verification, the mutation load can be modulated to reduce it to lower levels than those which lead to clinical manifestations. Diseases that involve mitochondrial defects or mt-nucleus signaling defects, but not the ones with nucleus involvement, will benefit from such therapies.

Challenges of Mitochondrial Transplantation

Although the studies summarized above show promising results in animal models, there are still many unknowns and challenges around mitochondrial transplantation, which need to be addressed. This could potentially also explain the reason behind the sparse number of clinical trials using mitochondrial transplantation.

One such challenge lies in the source of the mitochondria being used. Although most studies utilize autologous mitochondria, this greatly limits the clinical use of mitochondrial transplantation. In our opinion, fibroblasts may be the best source in terms of ease of access for mitochondrial transplantation for various purposes. However, the number of mitochondria in fibroblasts are not comparable to myocytes or cardiomyocytes. Platelets could potentially serve as another source; while platelets have a limited number of mitochondria, they are widely available. However, for more specialized cells such as those in the muscles, retina or cochlea, the mitochondria source may be restricted to the same cell type. The clinical usage of mitochondrial transplantation is also limited due to necessity and challenges of mitochondrial harvest. In clinical studies thus far, autologous mitochondria from either the abdominal rectus muscle or the pectoralis major muscles have been used and it has been reported that the activity of isolated mitochondria greatly decreases when stored on ice for more than one hour.(27) This short timeframe demands a rapid procedure, which may not be ideal for complex surgeries where timing is a crucial deterministic factor. However, if mitochondria could instead be used from a stored source instead of one that is needed to be rapidly extracted and prepared, clinical applications would be greatly broadened.

Another challenge for mitochondrial transplantation revolves around mtDNA haplotypes. mtDNA haplotypes are specific regions of mitochondrial DNA that cluster with other mitochondrial sequences and show origins of maternal lineages. mtDNA haplotypes are often associated with a variety of phenotypes and disease. Bevers et al.(100) outline that mitochondrial haplotypes have no direct impact on mitochondrial respiration, in drosophila, but may be associated with

metabolism-related phenotypes. Through swapping of mitochondrial genomes, they demonstrated that a mitochondrial haplotype associated with high food intake can rescue a low food intake phenotype, having drastic phenotypic implications.(100)

mtDNA haplotypes have been shown to influence mtDNA replication. Liu et al.(101) report that in swine models, different mitochondrial haplotypes are associated with different numbers of mtDNA copies. They also show that haplotypes with higher mtDNA copy numbers have an increased transcript abundance of mitochondrial-encoded subunits ND1, DN6 and ATP6. Ultimately, their data suggests that variations in mitochondrial haplotypes may affect mitochondrial content.(101) This is corroborated by findings from Lee at al.(102) They demonstrate that mtDNA haplotypes influence changes in gene expression and affinity for mtDNA replication factors to modulate mtDNA copy number. They also show that different haplotypes induce distinct DNA methylation patterns, which skew genetic expression patterns.(102)

It is evident that mitochondrial haplotypes may have drastic effects on genomic expression, and ultimately, phenotype. To our knowledge, we do not know of any studies that examines the effects of different mitochondrial haplotypes in the context of mitochondrial transplantation. Such studies are needed in the field, as transplanted mitochondria will conserve their DNA haplotype, and may affect respiratory capacity and metabolism.

Although sometimes practical, allogenic mitochondrial transplantation poses challenges of its own, particularly that it may mediate an autoimmune response in the host. The studies by McCully's group examined the role of immune system in response to mitochondrial transplantation, and several of their studies in animal models suggest that transplantation of autologous or allogenic mitochondria does not result in a significant alloreactivity or an increased Damage-Associated molecular pattern (DAMPs) response. Their results did not

suggest an increase in cytokine or chemokine levels, which are crucial mediators of immune responses.(44, 45, 48)

Conversely, other studies have shown that mitochondria may induce an inflammatory response. In fact, some DAMPS are associated with the mitochondria, likely secondary to its bacterial origin.(103-106) mtDNA, cytochrome C, and the mitochondrial enzyme carbamoyl phosphate synthase have all been found to induce inflammation through DAMPs.(103-106) Lin et al(107) demonstrated the ability of extracellular mitochondria to activate endothelial cells, resulting in an upregulation of endothelial cell adhesion molecules and the production of cytokines and chemokines. They also showed that the administration of allogenic mitochondria increased rejection of a xenograft heart transplant in a mouse model.(107) Pollara et al.(108) reported that mitochondria-associated DAMPs were abundant in the systemic circulation of post-mortem organ transplant donors. It was also concomitantly reported that pro-inflammatory cytokines and chemokines were elevated.(108) In the case of necrosis through direct tissue trauma, Zhang et al. reported that mitochondrial particles released during cellular necrosis can activate neutrophils, having immunostimulatory effects.(109, 110) These reports could pose a challenge to the therapeutic potential of mitochondria transplantation. More studies in the field are needed to examine the alloreactivity of allogenic mitochondria. Of particular use would be additional studies examining the mechanisms responsible for mitochondrial-mediated immune responses.

Conclusions

The studies summarized here collectively demonstrate the success and potential of mitochondrial transplantation in ameliorating mitochondrial function and ultimately in improving cardiac function. Despite advances in the field, challenges remain; the conceptual challenge is to better understand how mitochondrial transplantation affects intracellular processes, bioenergetics, and signaling. Additional studies are needed to investigate the role of mtDNA haplotypes and the potential for autoimmune reactions. Establishing the best route of access

and method of administering mitochondria to achieve the desired effect is of equal significance to institute mitochondrial transplantation as a viable clinical solution. Although direct injection of mitochondria is practical in surgical approaches, vascular access may serve as a more clinically appropriate mechanism of mitochondria distribution. Looking to the future, mitochondrial transplantation holds the potential to serve as a subcellular biotherapy to rejuvenate damaged or dysfunctional mitochondria and in doing so restoring homeostasis to the cells, protecting the heart tissue from damage, and ultimately improving cardiac function.

Author Contributions: P.A., S.H., and A.K. wrote and reviewed the manuscript. P.A. and S.H. prepared the figures, which were reviewed and approved by A.K.

Acknowledgements: We would like to thank Mathew Bell from Nanolive Microscope Team for their assistance with imaging studies that contribute to the image presented in Figure 3. Figures 1-2 and 5-7 were created on BioRender.com.

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878 **Figure Legands**

879 **Figure 1 | Mitochondrial isolation approaches in a glance.** Current mitochondrial isolation
880 techniques include **a.** Differential centrifugation (DC), **b.** Density gradient high-speed
881 ultracentrifugation (UC), **c.** Fractionated mitochondrial magnetic separation (FMMS), **d.** Isolation
882 for clinical use.

883 **Figure 2 | Mitochondrial transfer and internalization approaches.** Mitochondrial transfer
884 techniques include: **a.** Direct injection, **b.** Co-incubation, **c.** Centrifugation, **d.** Electromagnetic
885 forces (known as magnetomitotransfer), and e. photothermal (nanoblade).

886 **Figure 3 | Mitochondrial transplantation.** Non-autologous (allogenic) mitochondria from
887 skeletal muscle (rat L6; ATCC, Manassas, VA) cells were isolated using a commercially
888 available isolation kit (Thermofisher Scientific, Waltham, MA), labeled with MitoTracker Green
889 FM (Thermofisher Scientific, Waltham, MA) and transplanted to cardiomyocytes (rat H9c2;
890 ATCC, Manassas, VA) via combination of centrifugation and co-incubation. Success of
891 mitochondrial transplantation was visualized using holotomography microscopy (Nanolive SA,
892 Ecublens, Switzerland) 24 hours post-transplantation. The data is from Kheradvar Laboratory.

893 **Figure 4 | Native vs. transplanted mitochondrial population.** Mitochondrial transplantation
894 into cardiomyocytes visualized by structured illumination fluorescence microscopy (Keyence,
895 Itasca, IL). Transplanted mitochondrial population labeled with pHrodo Red SE (Thermofisher
896 Scientific, Waltham, MA), native mitochondria labeled with MitoTracker Green FM (Thermofisher
897 Scientific, Waltham, MA) and cell nucleus labeled with NucBlue Live (Thermofisher Scientific,
898 Waltham, MA). Figure adapted from an image previously published by Ali Pour et al. (2020).

899 **Figure 5 | Mitochondrial dysfunction in ischemia.** It is currently believed that irreversible
900 ischemic damage is due to: (1) loss of ATP, (2) supraphysiological free fatty acids (FFA) levels,
901 (3) formation of excess reactive oxygen species (ROS), (4) cellular calcium overload, and finally
902 (5) sodium pump inhibition.

Figure 6 | Mitochondrial dysfunction in mitochondrial diseases. Imbalance in mitochondrial dynamics (decrease in fusion, increase in fission, decrease in mitophagy) leads to accumulation of damage/mutant mitochondria (**Top**) and Clonal replication and expansion of damaged/mutant mtDNA beyond tissue threshold level for phenotype expression (**Bottom**).

Figure 7 | Mitochondrial Transplantation in Animal Models. Allogenic mitochondrial transplantation has been shown to increase cardioprotection in diabetic rat hearts (left), autologous mitochondria improve cardioprotection and decrease infarct size in rabbits undergone regional ischemia (middle), autologous mitochondria transplantation before ischemic damage in pigs leads to improved cardiac function and decreased infarct size, demonstrating mitochondrial transplantation's prophylactic effects (right). The diagrams summarize the work by Masuzawa et al. (2013), Doulamis et al. (2020), and Guariento et al. (2020).

Abbreviation	Phrase
mtDNA	Mitochondrial DNA
ROS	Reactive oxygen species
ETS	Electron transport system
DC	Differential Centrifugation
UC	Ultracentrifugation
MACS	Magnetic cell separation
FMMS	Fractionated mitochondrial magnetic separation
mECFP	Constitutively fluorescent enhanced cyan fluorescent protein
mTagRFP	Constitutively fluorescent red fluorescent protein
TAT-dextran	TransActivator of Transcription dextran
MbCD	Methyl-b-cyclodextrin
CytoD	Cytochalasin D
EIPA	5-(N-Ethyl-N-isopropyl) amiloride
Noco	nocodazole
EEA1	Early endosome antigen 1
FFA	Free fatty acid
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
MAPK	Mitogen-activated protein kinase
MCU	Mitochondria calcium uniporter
RYR-1	Ryanodine receptor 1
LETM1	Leucine zipper-EF-hand-containing transmembrane protein 1
NCLX	Na^{+}–Ca^{2+}–Li^{+} exchanger
LV	Left ventricular
DCD	Donation after circulatory death
PET	Positron emission tomography
ADH	Antidiuretic hormone
ECMO	Extracorporeal membrane oxygenation
DAMP	Damage-Associated molecular pattern

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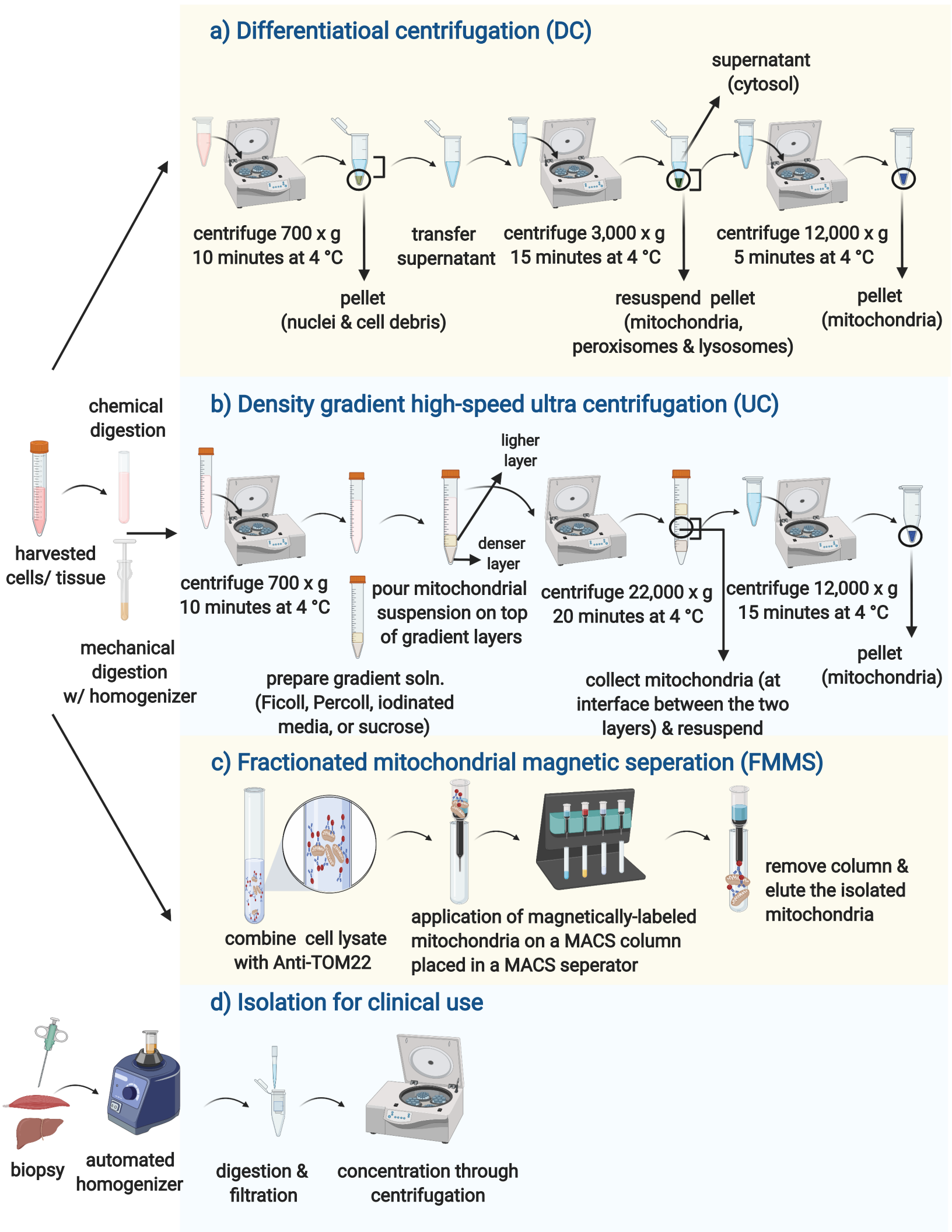
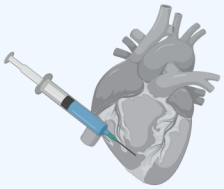
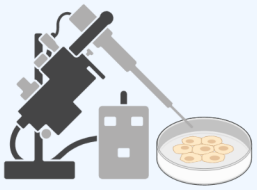

a) Differentiatioal centrifugation (DC)
supernatant (cytosol)
centrifuge 700 x g 10 minutes at 4 °C
transfer supernatant
centrifuge 3,000 x g 15 minutes at 4 °C
centrifuge 12,000 x g 5 minutes at 4 °C
pellet (nuclei & cell debris)
resuspend pellet (mitochondria, peroxisomes & lysosomes)
pellet (mitochondria)
chemical digestion
harvested cells/ tissue
mechanical digestion w/ homogenizer
b) Density gradient high-speed ultra centrifugation (UC)
ligher layer
denser layer
centrifuge 700 x g 10 minutes at 4 °C
pour mitochondrial suspension on top of gradient layers
centrifuge 22,000 x g 20 minutes at 4 °C
centrifuge 12,000 x g 15 minutes at 4 °C
pellet (mitochondria)
prepare gradient soln. (Ficoll, Percoll, iodinated media, or sucrose)
collect mitochondria (at interface between the two layers) & resuspend
c) Fractionated mitochondrial magnetic seperation (FMMS)
combine cell lysate with Anti-TOM22
application of magnetically-labeled mitochondria on a MACS column placed in a MACS seperator
remove column & elute the isolated mitochondria
d) Isolation for clinical use
biopsy
automated homogenizer
digestion & filtration
concentration through centrifugation

a) Direct Injection

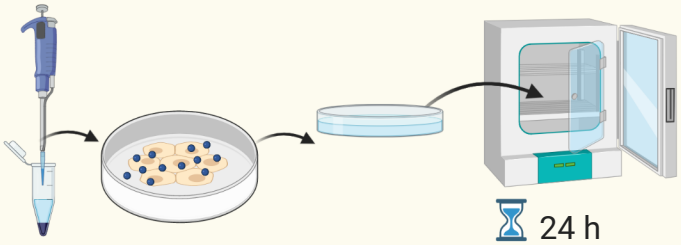


injection to affected tissue in animal model/clinical setting

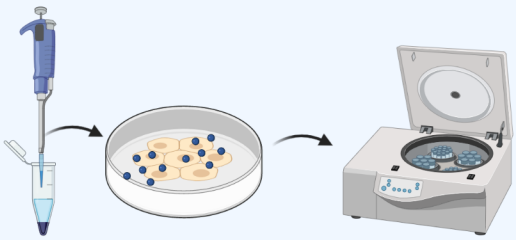


microinjection

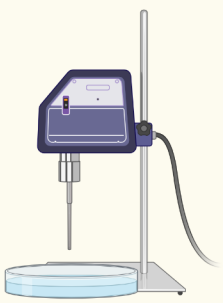
b) Co-incubation



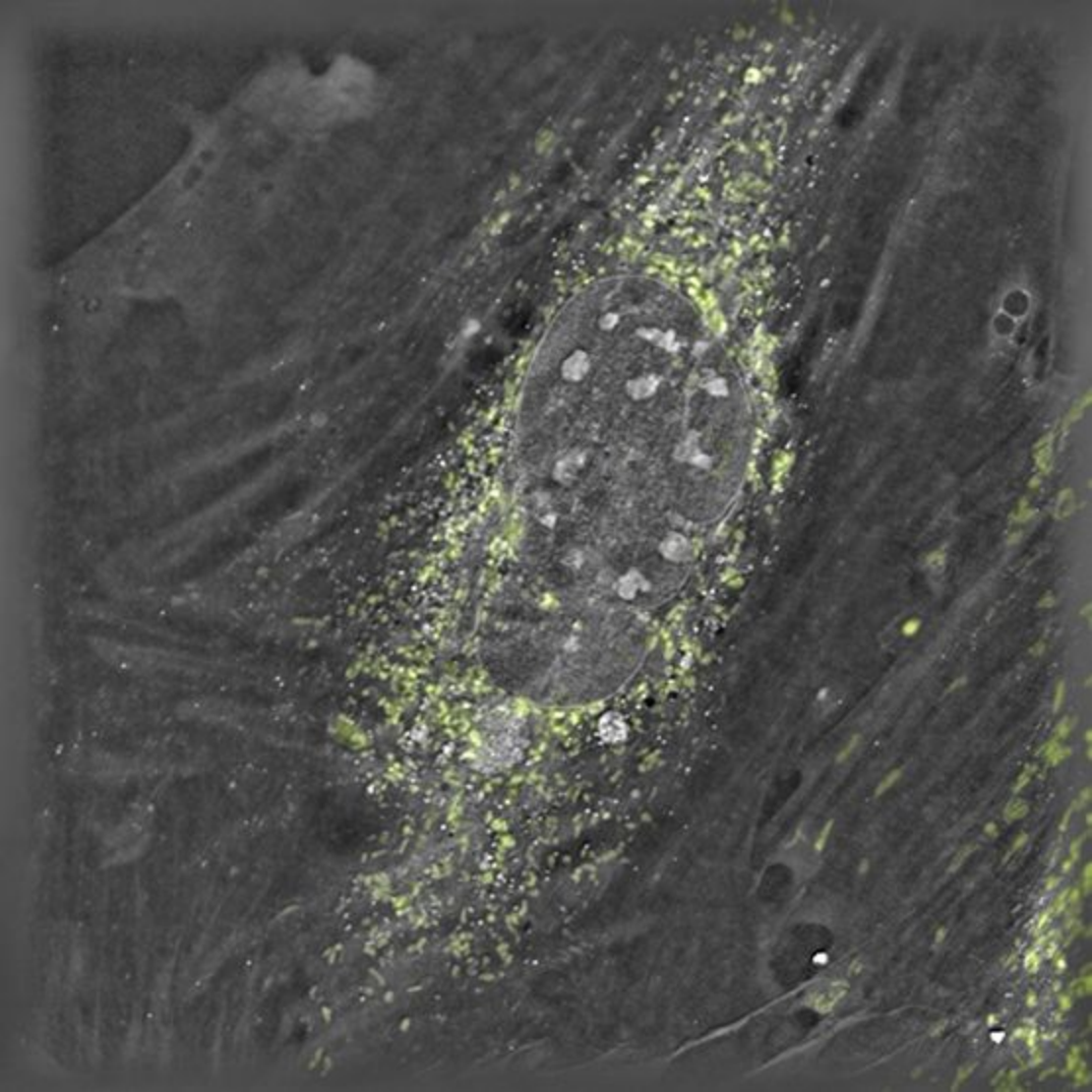
c) Centrifugation

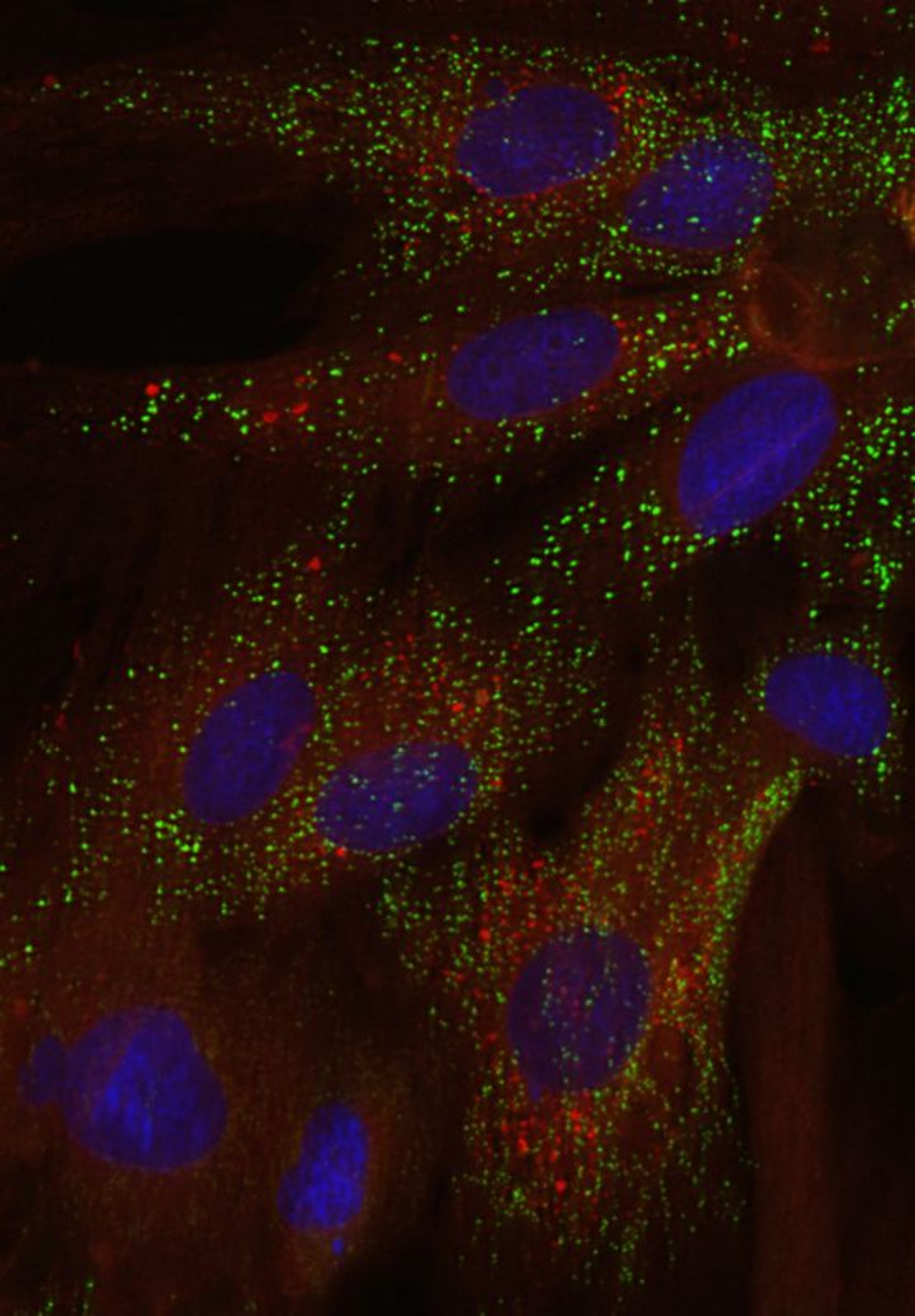


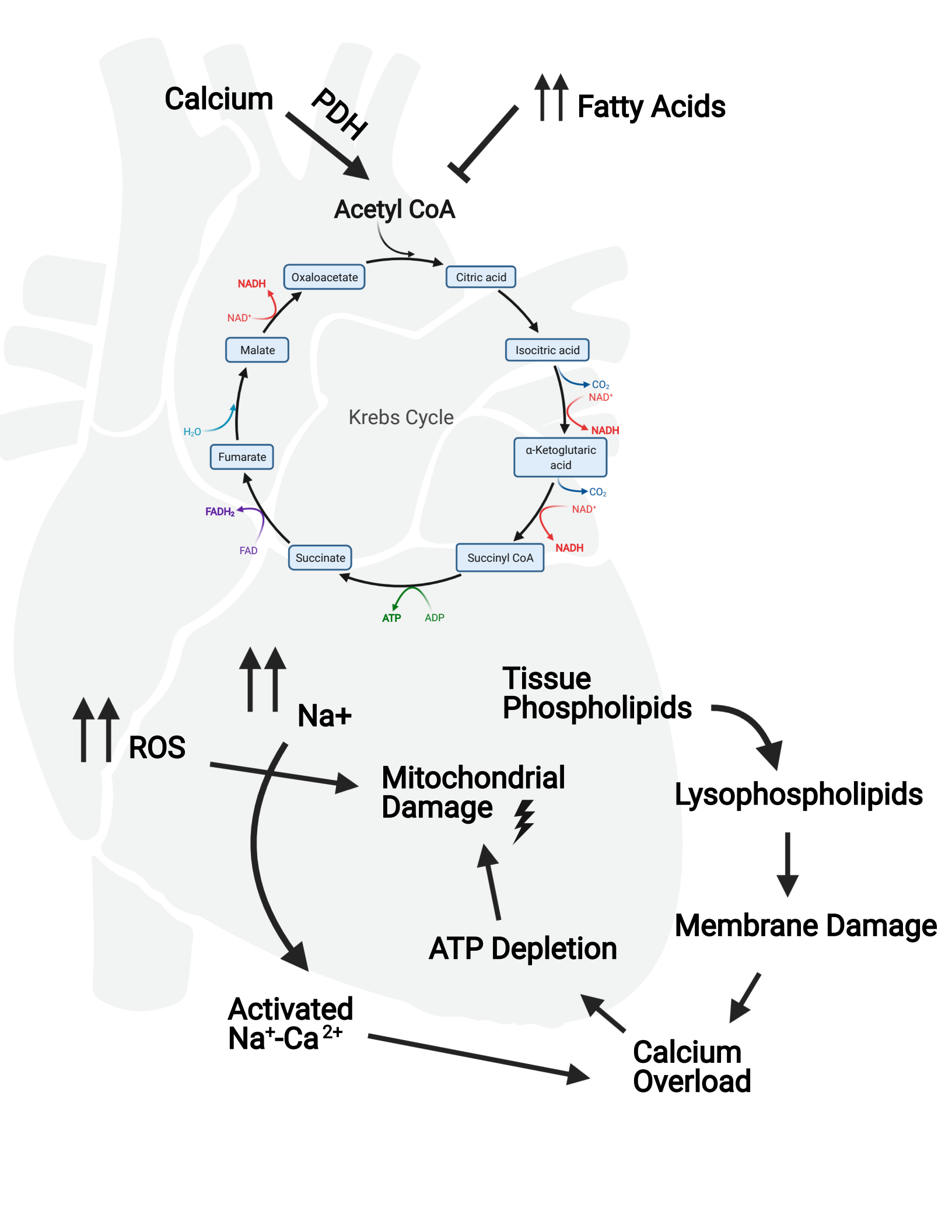
d) Electromagnetic Forces (Magnetomitotransfer)

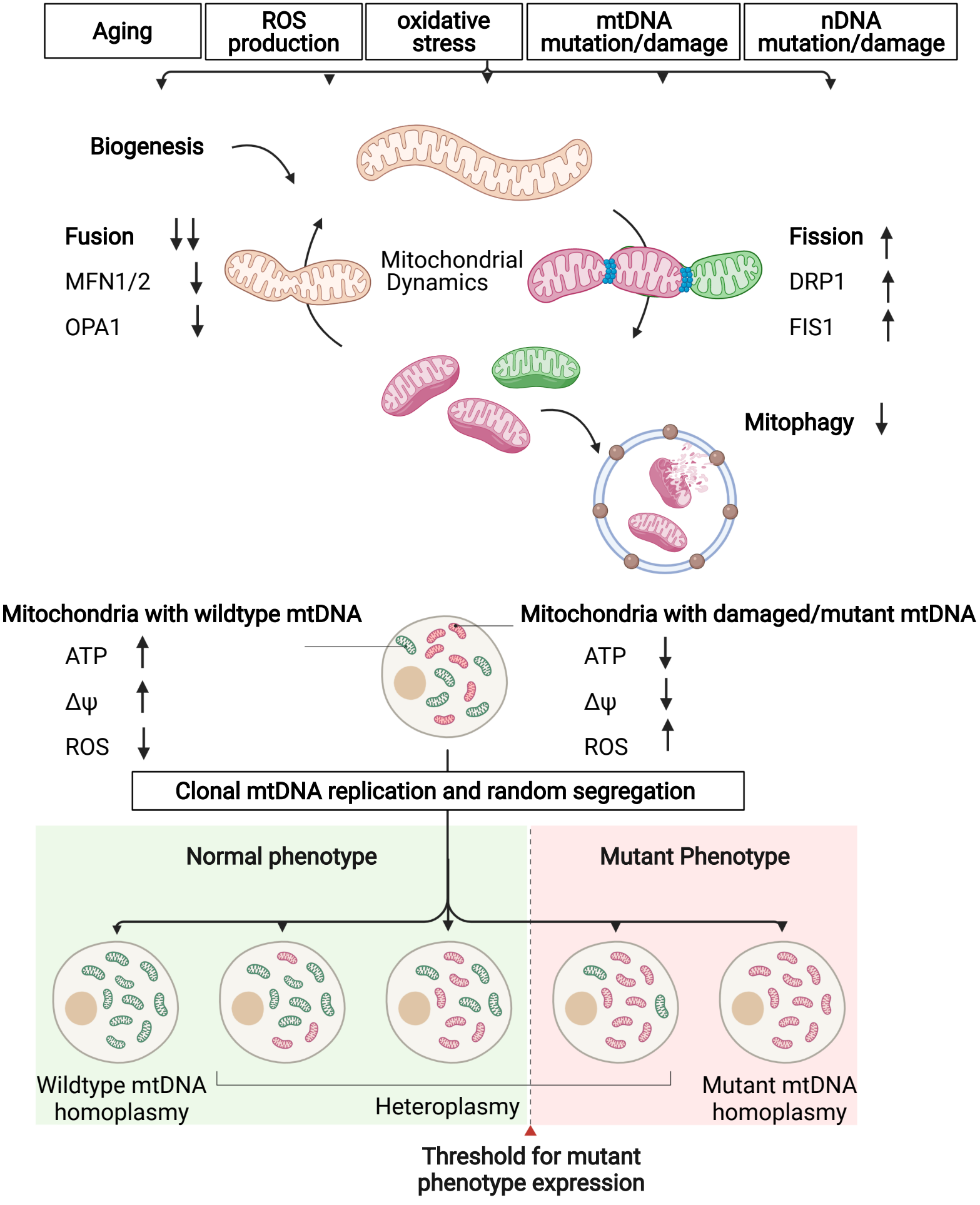


e) Photothermal (Nanoblade)






Calcium
PDH
Fatty Acids
Acetyl CoA
Oxaloacetate
Citric acid
NADH
NAD+
Malate
Isocitric acid
CO2
NAD+
NADH
Krebs Cycle
H2O
Fumarate
α-Ketoglutaric acid
CO2
NAD+
FADH2
FAD
NADH
Succinate
Succinyl CoA
ATP
ADP
Na+
ROS
Tissue Phospholipids
Mitochondrial Damage
Lysophospholipids
Membrane Damage
ATP Depletion
Activated Na+-Ca2+
Calcium Overload

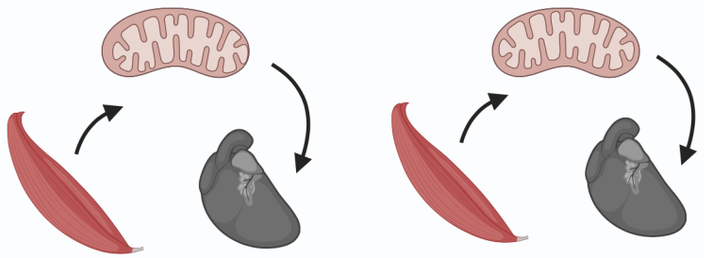

Aging
ROS production
oxidative stress
mtDNA mutation/damage
nDNA mutation/damage
Biogenesis
Fusion
MFN1/2
OPA1
Mitochondrial Dynamics
Fission
DRP1
FIS1
Mitophagy
Mitochondria with wildtype mtDNA
ATP
Δψ
ROS
Mitochondria with damaged/mutant mtDNA
ATP
Δψ
ROS
Clonal mtDNA replication and random segregation
Normal phenotype
Mutant Phenotype
Wildtype mtDNA homoplasmy
Heteroplasmy
Mutant mtDNA homoplasmy
Threshold for mutant phenotype expression

Diabetic rat hearts are subjected to 30 mins of global ischemia

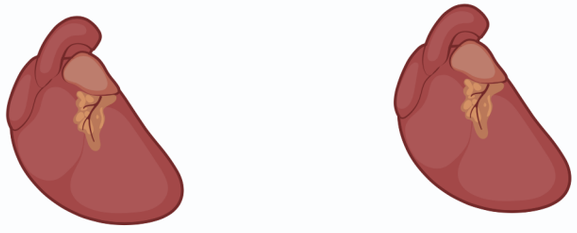
Diabetic rat hearts recieve allogenic mitochondria from either non-diabetic rat skelatal muscle or diabetic rat skelatal muscle

<u>Non-diabetic</u>

<u>Diabetic</u>



120 min reperfusion



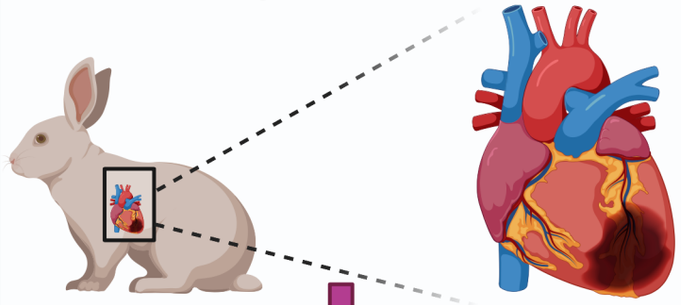
Cardioprotection:

- ↑ cardiac function
- ↓ infarct size

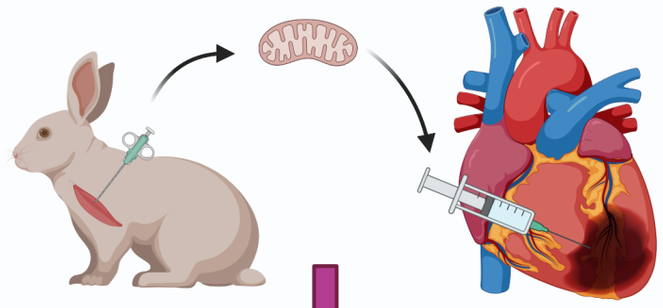
Cardioprotection:

- ↑ cardiac function
- ↓ infarct size

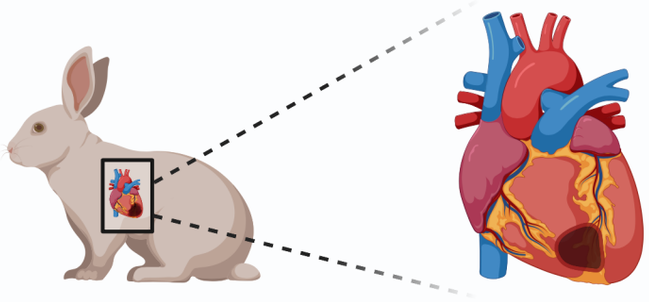
Rabbits subjected to 29 mins of regional ischemia



Rabbits recieve autlogous mitochondria from pectoralis muscle



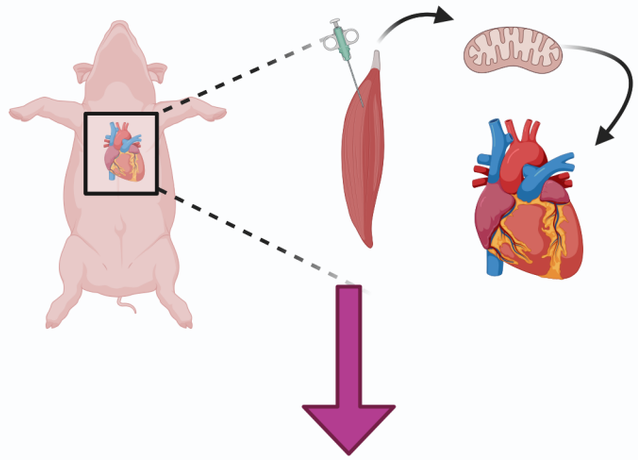
Rabbits recover for 4 weeks



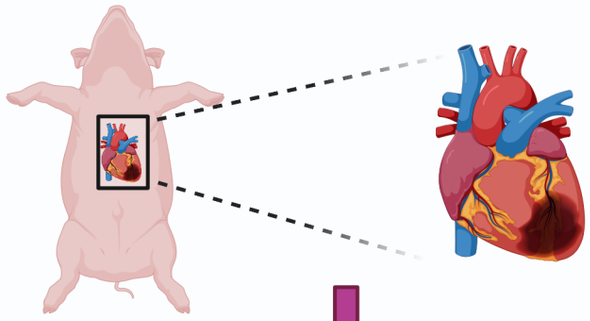
Cardioprotection:

- ↓ Creatine Kinase
- ↓ apoptosis
- ↓ infarct size
- ↑ cardiac function

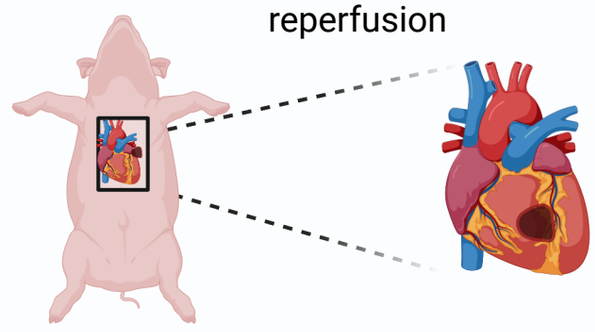
Pigs recieve autologous mitocondria pre-ischemia



Pigs undergo regional ischemia for 30 minutes



120 min reperfusion



Cardioprotection:

- ↑ coronary blood flow
- ↑ cardiac function
- ↓ infarct size
- ↓ myocardial strain