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PERSPECTIVE

Mitochondrial transplantation as a promising therapy for mitochondrial diseases

Tian-guang Zhang, Chao-yu Miao*

Department of Pharmacology, Second Military Medical University/Naval Medical University, Shanghai 200433, China

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Ethical issue

Abstract Mitochondrial diseases are a group of inherited or acquired metabolic disorders caused by mitochondrial dysfunction which may affect almost all the organs in the body and present at any age. However, no satisfactory therapeutic strategies have been available for mitochondrial diseases so far. Mitochondrial transplantation is a burgeoning approach for treatment of mitochondrial diseases by recovery of dysfunctional mitochondria in defective cells using isolated functional mitochondria. Many models of mitochondrial transplantation in cells, animals, and patients have proved effective *via* various routes of mitochondrial delivery. This review presents different techniques used in mitochondrial isolation and delivery, mechanisms of mitochondrial internalization and consequences of mitochondrial transplantation, along with challenges for clinical application. Despite some unknowns and challenges, mitochondrial transplantation would provide an innovative approach for mitochondrial medicine.

*Corresponding author. Tel: +86 21 81871271; fax: +86 21 65493951.
E-mail address: cymiao@smmu.edu.cn (Chao-yu Miao).

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1. Introduction

Mitochondria, the vital organelles of eukaryotic cells, are integrators of various cellular metabolic pathways, including oxidative phosphorylation, fatty acid oxidation, urea cycle, Krebs cycle, ketogenesis and gluconeogenesis[1]. Mitochondria are also important in many other essential cellular processes such as calcium homeostasis, lipid metabolism, amino acid metabolism, biosynthesis of heme, and thermogenesis[2]. However, they also have important roles in many pathways which can cause both apoptosis and necrosis[3]. Therefore, the importance of the mitochondrion in the maintenance of cellular homeostasis is well established, meanwhile a large amount of evidence shows that mitochondrial dysfunction is deleterious[4].

Due to the essential function of mitochondria in the human body, mitochondrial dysfunction causes a great variety of mitochondrial diseases, which can affect almost all the organs in the body and present at any age[4,5]. Mitochondrial diseases are a group of metabolic disorders characterized by energy metabolism dysfunction. The pathophysiology is further complicated by the involvement of genetic mutations in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) which encode mitochondrial proteins. This means that mitochondrial diseases may result from inheritance for nDNA mutations and maternal inheritance for mtDNA mutations. The estimated minimum prevalence of mitochondrial diseases is 1 in 5000, whereas it could be higher[6].

As advances in molecular and biochemical methodologies led to a better understanding of the mechanisms of mitochondrial disorders for various diseases, mitochondria have become a major target for research institutions and pharma companies. Pharmacological approaches include dietary supplements such as agents increasing respiratory chain function (coenzyme Q10 and riboflavin), agents inducing mitochondrial biogenesis (AICAR and bezafibrate), antioxidants (vitamin C and vitamin E), mitochondrial substrates (L-carnitine) and so on[7,8]. However, these agents fail to significantly alleviate disease symptoms or effectively slow disease progressions, there has therefore been no satisfactory therapeutic strategy available for mitochondrial diseases so far[9]. In addition, all new drugs under clinical trials for treatment of mitochondrial diseases are unable to cure these diseases permanently[9]. For these reasons, therapies using the above agents have limitations.

Natural mitochondrial transfer between cells realizes intercellular energy synchronization[10–13]. Intercellular mitochondrial transfer can occur *via* intercellular structures such as tunneling nanotubes (TNTs), dendrites and secreted cellular bodies such as microvesicles as well as release and internalization of naked mitochondria[14] (Fig. 1). TNTs are membranous tubular protrusions between adjacent cells for cellular component exchange and cell-to-cell communication[15]. TNTs are regarded as the main mitochondrial transfer between cells[16]. Dendrite is another form of membranous protrusion connected to form intercellular networks. Some cells such as osteocytes are connected to each other by their intrinsic dendrites to facilitate intercellular mitochondrial transfer[17]. According to their origins, extracellular vesicles, a population of secreted membrane vesicles, are mainly divided into three types with different sizes which include exosomes (30−100 nm), microvesicles (100 nm−1 μm), and apoptotic bodies (>1 μm)[18]. Microvesicles are formed by direct plasma membrane blebbing which can encapsulate organelles including mitochondria due to their larger diameters and therefore participate in mitochondria transfer for long-distance intercellular communication[19]. Mitochondria can be released not only encapsulated by a bilipid membrane but also naked. Naked mitochondria without carriers can be extruded and internalized between cells. Recently, naked respiratory competent mitochondria were reported in circulating blood[20]. Although further research is needed to fully understand the role of the naked mitochondria released by cells, artificial mitochondria transfer approaches in order to transfer intact mitochondria into recipient cells have been developed[19].

2. Mitochondrial transplantation

Mitochondrial transplantation is a burgeoning approach for treatment of mitochondrial diseases by recovery of dysfunctional mitochondria in defective cells with isolated functional mitochondria. For the limitations of therapies using agents, mitochondrial transplantation can offer exciting therapeutic applications in mitochondrial medicine. In addition to the intrinsic intercellular mitochondrial transfer, mitochondria are far from static and their morphologies continually change by the combined actions of fusion and fission within cells (Fig. 2). Mitochondria are usually defined as rounded or elongated, where they can fuse and interconnect into networks. Fission rescues stress by removal of damaged mitochondria and fusion helps to complement dysfunctional mitochondria by sharing components from functional mitochondria[21–25]. Mitochondrial transplantation indicates that exogenous mitochondria are able to fuse with endogenous mitochondria in recipient cells, along with an increase in oxygen consumption rates, ATP content, and replacement of depleted mitochondrial DNA[26,27]. Mitochondrial transplantation includes isolation, delivery, and internalization of exogenous functional mitochondria into the target cell or tissue (Fig. 3).

2.1. *Isolation*

2.1.1. *Mitochondrial source*

Generally, almost any normal tissue or cell which are far from the lesion area can be used as a source of isolated mitochondria. For clinical application, autologous skeletal muscles, such as rectus abdominis muscle[28], pectoralis major muscle[29], gastrocnemius muscle[30] and so on, are good sources for mitochondria isolation in order to enhance clinical practicality and reduce immunogenic complications as much as possible. Although other healthy tissue sources can also be used, different number of mitochondria were provided by different tissue sources as the absolute number of mitochondria in liver is the greater than skeletal muscle, followed by cardiac muscle[31].

2.1.2. *Mitochondrial isolation approach*

Mitochondria isolation can be obtained using techniques such as differential centrifugation[32] and density gradient centrifugation[33]. Some bad mitochondria such as swollen mitochondria (*e.g.*, after permeability transition) have a decrease in density, thus isolation of mitochondria by these two methods may select intact mitochondria by elimination of the bad mitochondria with lower density. However, yield is higher and purity is lower in differential centrifugation[34], whereas yield is lower and purity is higher in density gradient centrifugation. In addition, these mitochondrial isolation methods need time consuming, repetitive centrifugation steps, resulting in decreased mitochondrial viability[35].

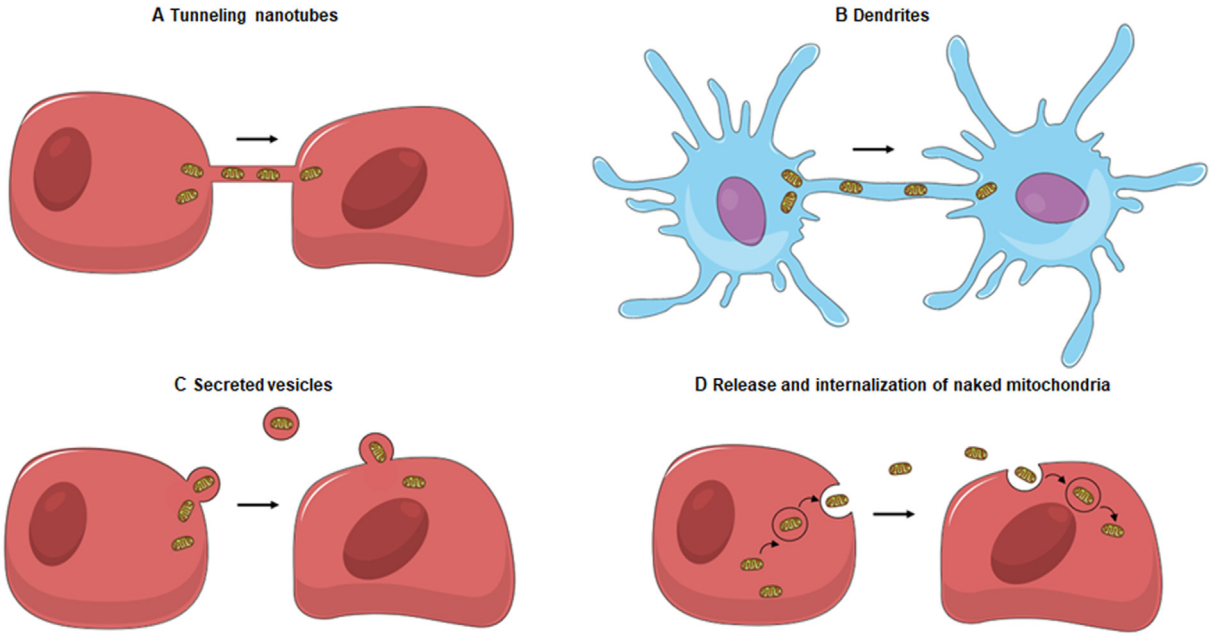

Figure 1 Natural mitochondrial transfer.

Protocols to isolate mitochondria from different sources including tissues and cells based on differential centrifugation have been developed and each protocol is best suited only for isolation of mitochondria from the specific source[36]. Take isolation of mouse skeletal mitochondria for example. Briefly, the minced skeletal muscle tissues were digested in EDTA and trypsin on ice for 30 min. After centrifugation of the digested tissue at 200 × *g* for 10 min at 4 °C, the pellet of the tissue was homogenized in the ice-cold isolation buffer. And the homogenate was centrifuged at 700 × *g* for 10 min at 4 °C. Then the supernatant was collected and centrifugated at 8000 × *g* for 10 min at 4 °C for mitochondria pelletting. Lastly, the pellet containing mitochondria was washed with ice-cold isolation buffer and centrifuged again at 8000 × *g* for 10 min at 4 °C before further application.

Density gradient centrifugation is often used for isolation of brain mitochondria with very low contamination from synaptosomes and myelin[33,37]. In brief, the tissue was homogenized in the ice-cold isolation buffer and the homogenate was centrifuged at 1330 × *g* for 3 min at 4 °C. The supernatant was centrifuged at 21,000 × *g* for 10 min at 4 °C. And the pellet was resuspended in cold 15% gradient Percoll solution. This material was slowly poured on the discontinuous density gradient layers consisting of 23% Percoll above of 40% Percoll in the centrifuge tube. After centrifuging the tube at 30,700 × *g* for 5 min at 4 °C, three distinct bands of material were obtained and the material banding between the interface of the lower two Percoll layers containing the highly enriched mitochondrial fraction was diluted with isolation buffer and centrifuged at 16,700 × *g* for 10 min at 4 °C to produce a pellet consisting of mitochondria.

A rapid method for isolating and purifying mitochondria recently proposed by McCully's team may better meet the requirements for clinical application where an interventional time is less than 30 min[38]. The major benefit of this method is that the use of differential filtration in place of differential centrifugation which allows for more rapid isolation of highly purified, viable and intact mitochondria. The isolated mitochondria are shown to

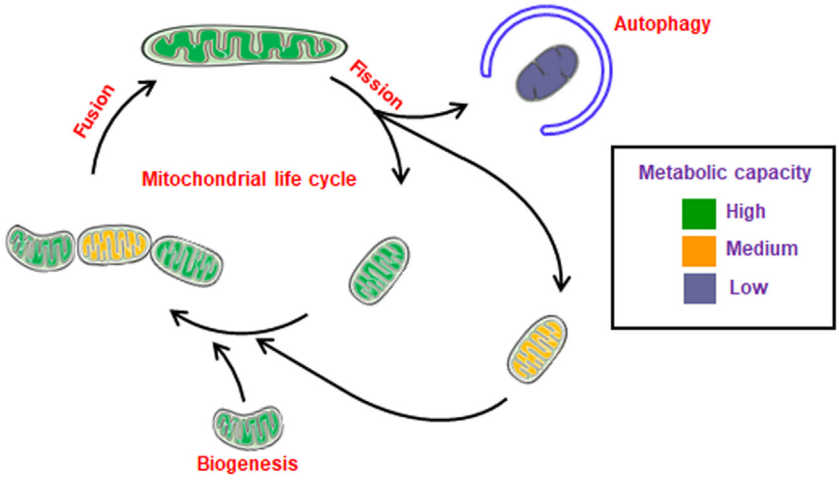

Figure 2 Mitochondrial life cycle. The mitochondrial life cycle begins with growth of the pre-existing healthy mitochondria by biogenesis and ends with removal of unhealthy mitochondria by autophagy, between which mitochondria continuously undergo fusion and fission.

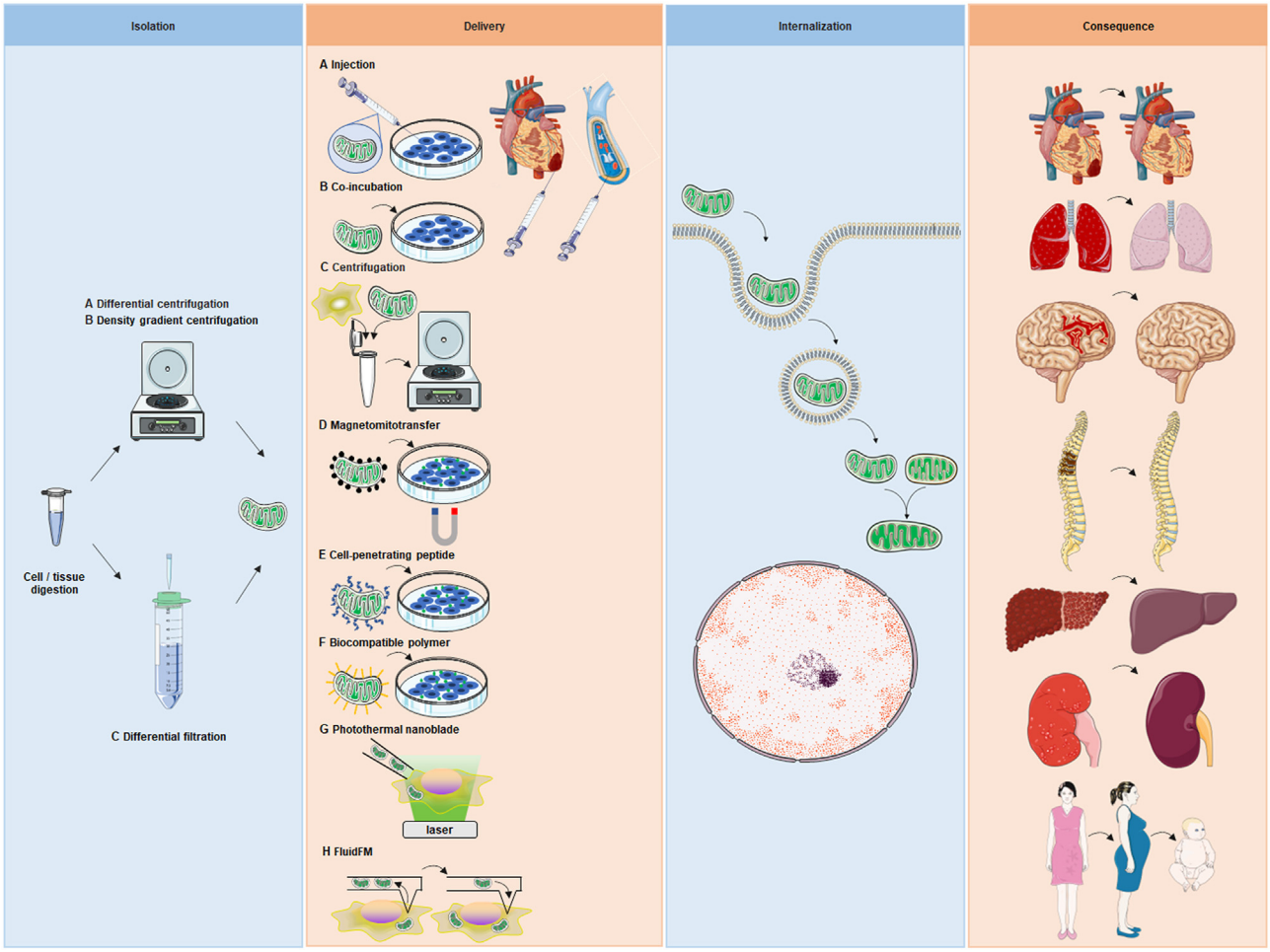

Figure 3 Schematic illustrating mitochondrial transplantation.

be less than 0.01% being fractured or damaged and less than 0.001% contamination of non-mitochondrial particles[38]. In brief, two small pieces of tissue are collected using a 6 mm biopsy sample. The tissues are homogenized in isolation buffer using a commercial automated homogenizer for 1 min and digested by Subtilisin A on ice for 10 min. The digested homogenate is successively filtered through a pre-wetted 40 μm mesh filter, followed by a new pre-wetted 40 μm filter, followed by a new pre-wetted 10 μm filter and these three-time filtrations require 2−3 min. The purified mitochondria can be used directly or further concentrated by centrifugation at 9000 × *g* for 10 min at 4 °C.

2.1.3. *Mitochondrial evaluation*

Viable mitochondria are required for mitochondria transplantation. Mitochondrial function can be easily and rapidly assessed by fluorescence microscope using mitochondrial membrane potential fluorescent probes such as TMRM, TMRE and MitoTracker red[39]. Viable, respiration competent mitochondria can be stained with these probes.

In addition, MitoTracker Green is another mitochondrial fluorescent probe labeling all mitochondria independent of mitochondrial membrane potential. The use of MitoTracker Green in combination with any of these mitochondrial membrane potential fluorescent probes above allows for identification of the amount of viable ones in all mitochondria within a preparation. Besides, oxygen consumption rate examined by Clark type electrodes and ATP content measured by ATP luminescence assay can further identify the mitochondrial function by assessment of coupled respiration or oxidative phosphorylation.

The isolated mitochondria must be pure as far as possible and therefore they should be substantially free of cytoplasmic and nuclear contaminants. Western blot can be used to assess mitochondrial purity by antibodies targeting specific contaminants such as cytosol by anti-GAPDH, nucleus by anti-histone H3, Golgi by anti-GM130 and so on. Electron emission microscopy is another valuable method to analyze isolated mitochondrial structure and purity, whereas it is not suitable for rapid evaluation.

For clinical application, Good Manufacturing Practice should be established in order to ensure that the quality of isolated mitochondria meets requirements of all the assays described above. In this case, mitochondrial fluorescent probe staining may be enough to rapidly confirm mitochondrial quality following each isolation at a minimum, for it can be performed in 5−10 min, with evaluation for about 5−10 min.

2.2. *Delivery*

The transplantation of isolated mitochondria from exogenous sources into different recipient cells has been successful in multiple models *in vitro* and *in vivo*. Incorporation of exogenous mitochondria to recipient cells has been attempted by direct injection, co-incubation, centrifugation, magnetomitotransfer, cell-

penetrating peptide, biocompatible polymer, photothermal nanoblade and fluidic force microscope (FluidFM).

Direct injection has been used to directly deliver isolated mitochondria into recipient cells by microinjection or tissues through a needle in models *in vivo* and *in vitro* including several clinical trials[28,29,31,40]. In addition to local injection to the tissue, *in vivo* direct injection can be completed by systemic injection *via* vein[41]. *In vivo*, local injection of mitochondria is useful in local damage, whereas intravascular injection is more effective for multiple organ mitochondrial diseases.

Co-incubation of isolated mitochondria with recipient cells has been successful to deliver exogenous mitochondria into cells *in vitro*. As mitochondria are originated from bacteria, these are similar to the way that bacteria are taken up by eukaryotic cells. Twice centrifugations of the culture plates at 1500 × *g* for 15 min at 4 °C before and after co-incubation for the first 2 h can enforce the delivery of isolated mitochondria into the recipient cells[42]. Co-incubation is simple, but it is unsuitable for *in vivo* mitochondrial delivery.

Centrifugation of isolated mitochondria and recipient cells at 1500 × *g* for 5 min at 4 °C in a microcentrifuge tube is a simple and quick method to deliver exogenous mitochondria into culture cells without further co-incubation[43]. This strategy is a good way to delivery of mitochondria into the target cell without membrane disruption or technical requirements like microinjection, photothermal nanoblade and FluidFM, whereas it cannot be used for *in vivo* mitochondrial delivery.

Magnetomitotransfer is a technique using magnetic beads to bind to mitochondrial outer membrane protein TOM22[44]. This technique can increase mitochondrial internalization into 78%−92% recipient cells, compared with 17% for co-incubation groups. However, TOM22 exists in not only functional mitochondria but also dysfunctional mitochondria and therefore the dysfunctional mitochondria may also be simultaneously delivered into the cells. Besides, the possible toxicity of magnetic beads and unwanted materials is unknown and this method is not applicable for *in vivo* mitochondrial delivery.

Pep-1, a cell-penetrating peptide, is used to conjugate with mitochondria to promote the cellular uptake of the isolated mitochondria. More than 75% Pep-1 conjugated mitochondria can be internalized into recipient cells[45], whereas cellular uptake ability of naked mitochondria is low. As Pep-1 promotes translocation of cargo such as small peptides and DNA independent of endosomal pathway[46], isolated mitochondria which were delivered into cells by electrostatic and hydrophobic contacts of Pep-1 with the cell membrane may directly participate in recovery of dysfunctional mitochondria without escape from endocytic vesicles. The Pep-1-conjugated mitochondria promotes neuron survival and movement recovery of Parkinson's disease rats compared with naked mitochondria[47], whereas the immunogenicities and cytotoxicities of different cell-penetrating peptides including Pep-1 should be further identified individually[48] and this approach cannot be used for *in vivo* mitochondrial delivery.

Dextran is a natural polysaccharide with good biocompatibility and has proven advantageous for mitochondria delivery. Dextran-triphenylphosphonium coated mitochondria protect their respiratory function and facilitate their cellular internalization compared with uncoated mitochondria[49]. However, this method is unapplicable for *in vivo* mitochondrial delivery.

Photothermal nanoblade is a technique for transfer of isolated mitochondria into cells *via* a titanium coated micropipette which can be rapidly heated by a laser pulse to open the cell membrane[50]. This method has a mitochondrial transfer efficiency of 2% which is higher than microinjection (0.2%−0.3%)[50,51]. Nonetheless, this method is low throughput and needs equipment to execute the procedure and cannot be used *in vivo*.

FluidFM is a recently established approach to extract, inject, and transplant mitochondria at high efficiency between single living cells using a FluidFM cantilever without compromising mitochondria integrity nor their viability, due to no need for the complex processes of mitochondrial isolation used in other delivery strategies mentioned above[52]. Unlike other strategies requiring the complex processes of mitochondrial isolation, FluidFM enables mitochondria to experience short extracellular time (< 1 min) and to be maximally concentrated in native cytosol rather than artificial solutions, ensuring mitochondria integrity and their viability. High transfer efficiency of 95% can be achieved by FluidFM, which is much greater than that of microinjection or photothermal nanoblade mentioned above. However, equipment was needed to successfully carry out the procedure and this method reduces throughput and still cannot be used *in vivo* either.

In brief, direct injection involving local injection and intravenous injection can be used for delivery of mitochondria *in vivo*. For *in vitro* mitochondrial delivery, approaches including centrifugation, magnetomitotransfer, cell-penetrating peptide and biocompatible polymer have been developed to further improve mitochondrial internalization, compared with co-incubation. Nevertheless, invasive methods including microinjection, photothermal nanoblade and FluidFM are all less effective than co-incubation, due to their limited transplanted cell numbers and possible damage of the recipient cells.

2.3. *Internalization*

Mitochondrial internalization has been observed in living cells. The integrity of mitochondrial outer membrane and viability of the mitochondria are necessary for mitochondrial internalization[53,54]. Mitochondrial uptake involves several possible mechanisms such as caveolae-dependent-clathrin dependent endocytosis[55], actin-meditated endocytosis[27] and macropinocytosis[56]. A recent study using three-dimensional super-resolution microscopy and transmission electron microscopy demonstrated that the isolated mitochondria were incorporated into recipient cells and then transported to endosomes and lysosomes[26]. The majority of internalized mitochondria escape from the endolysosomal system and then fuse with the endogenous mitochondrial network[26]. More than one mechanism may be involved in internalization of exogenous mitochondria by recipient cells and thus more studies are needed to identify additional mechanisms of mitochondrial internalization.

2.4. *Consequence*

Many reports have shown that various models of mitochondrial transplantation in cells, animals, and patients, regardless of autologous or non-autologous, have proved effective. This therapeutic intervention has been confirmed to be successful for treating myocardial ischemia−reperfusion injury, cardiogenic shock following ischemia−reperfusion injury and repeated failure infertility in clinical trials. McCully's team performed the first clinical application of autologous mitochondrial transplantation which is useful for pediatric patients suffering from myocardial ischemia−reperfusion injury after cardiac surgical procedure[31]. Of the 5 subjects, 4 demonstrated cardiac functions improved and were successfully removed from extracorporeal membrane oxygenation

(ECMO) support. A recent retrospective study of pediatric patients with severe refractory cardiogenic shock due to myocardial ischemia−reperfusion injury after cardiac surgery also showed that autologous mitochondrial transplantation was associated with successful separation from ECMO and improvement of cardiac function overall[28]. Eight in ten patients undergoing revascularization followed by mitochondrial transplantation were successfully free from ECMO, whereas only 4 in 14 patients undergoing revascularization alone became ECMO free. In China, Liang's team[57] performed autologous mitochondrial transplantation in oocytes for treatment of an infertile patient undergoing repeated failure in fertilization *in vitro* and the patient successfully obtained a live baby boy. There has also been reported to be useful in animal studies partly with cell models for the treatment of mitochondrial defect in organs involving heart, lung, brain, bone, liver, and kidney, which include myocardial ischemia−reperfusion injury[58], acute respiratory distress syndrome[59], Parkinson's disease[60], spinal cord injury[61] , liver injury[62] and acute kidney injury[63] (Fig. 3). Mitochondria isolated from allogeneic myocardial tissue were directly into the injury regions of ischemic rabbit hearts shortly before reperfusion resulting in significant enhancement in cardioprotection with increased ATP content, reduced infarct size and improved postischemic myocardial function[58]. Allogeneic liver-derived mitochondria alone or together with melatonin could protect the lungs from acute respiratory distress syndrome in a rat model by the inhibition of ROS generation, DNA damage, inflammation and apoptosis[59]. Treatment with mitochondria isolated from human HepG2 cells reduced oxidation stress besides increase of energy supply in an *in vitro* Parkinson's disease model and Parkinson's disease mice systemically administrated with isolated mitochondria from HepG2 also show improved pathological progression as evidenced by decreased ROS level, increased activity of electron transport chain and down-regulated apoptosis and necrosis, together with improved locomotor activity[60]. In a spinal cord injury of rat model, mitochondria isolated from either cultured cells or allogeneic muscles could maintain the cellular bioenergetics in contused spinal cord of the rats and improve their locomotor activity[61]. For *in vitro* and *in vivo* models of APAP-induced liver injury, exogenous mitochondria from HepG2 cells decreased oxidation stress, increased energy supply, and reduced hepatotoxicity[62]. It was reported that autologous mitochondria isolated from sternocleidomastoid muscle increased estimated glomerular filtration rate and urine output, while decreased serum creatinine and blood urea nitrogen, resulting in only patchy mild acute renal tubular injury in an acute kidney injury of swine model[63].

All these clinical and animal studies suggest the potential of mitochondrial transplantation in various mitochondrial diseases. Furthermore, according to https://clinicaltrials.gov, 3 clinical trials of autologous or allogeneic mitochondria transplantation are now underway, which deal with patients suffering from myocardial ischemia/reperfusion injury, cerebral ischemia injury and refractory polymyositis/dermatomyositis.

3. Challenges

Although mitochondrial transplantation has shown to be a promising future, there are still many unknowns and challenges needed to be addressed for its clinical application. The major challenges include mitochondrial storage, transplantation rejection, and ethical issues.

It has been reported the isolated mitochondria can stay active when stored on ice for approximately 1−2 h[35,64]. Application range of mitochondrial transplantation is limited by the narrow time window of mitochondrial isolation. The isolation of mitochondria must be timely and the short time frame demands a rapid procedure which is not suitable for time-consuming surgeries. Thus, the rapid isolation and purification of mitochondria by differential filtration is preferred, compared with differential centrifugation and density gradient centrifugation. However, if isolated mitochondria can be used as a storable preparation instead of preparation for each time, clinical applications of mitochondrial transplantation would be greatly broadened. Therefore, establishment of a method that allows mitochondria to be stored for a long time is very important.

Transplantation of autologous mitochondria would prevent inflammation and rejection as they are derived from the patients' own body. In an animal model, autologous mitochondrial transplantation caused no significant increase in sensitive markers of inflammation such as TNFα, IL-6 and high-sensitivity C-reactive protein and no anti-mitochondrial antibody was detected either[31]. In addition, multiplex analysis of cytokines and chemokines in human peripheral blood mononuclear cells has confirmed that there are no autoimmune or inflammatory response associated with autologous mitochondrial transplantation[31]. However, for patients with congenitally mitochondrial diseases, autologous mitochondria transplantation would not be suitable as inherently defective mitochondria may exist in other tissues. In these cases, mitochondria from a different individual of the same species are required. A study has shown that allogeneic mitochondria did not induce alloreactivity, allorecognition or damage-associated molecular patterns reaction[65]. Conversely, other studies have shown that allogenic mitochondria may induce inflammatory response. Damage-associated molecular patterns derived from circulating mitochondria in deceased organ donation is associated with allograft dysfunction, indicating a role for circulating extracellular mitochondria in allograft outcomes[66]. Further study showed that allogenic mitochondria were able to activate human endothelial cells *in vitro*, resulting in increase of adhesion molecules, inflammatory cytokines and chemokines[67]. Thus, more studies on the outcomes and mechanisms responsible for immune response of allogenic mitochondrial transplantation are required to greatly broaden the clinical applications.

Mitochondrial transplantation involving the transfer of mtDNA but not any kind of nuclear material has great potential to satisfy ethical concerns, as the genes deciding personal traits come from nDNA rather than mtDNA[68]. Autologous mitochondria from the tissue with a low mtDNA mutation risk could be used to treat the same person and have few ethical concerns. Though the mtDNA constitutes only 0.1% of the total DNA and the sequence variation among different mitochondrial haplotypes in the human being is low[69], mitochondrial allotransfer involves the transfer of mtDNA from one person to another and thus the ethical concerns are needed to be addressed. For allotransfer, to reduce mtDNA differences, mitochondria from genetically close family members should be firstly considered and if not, haplotype matching could be considered[70,71]. More details regarding the ethical issues of mitochondrial transplantation are described in the other review article[19].

4. Conclusions

As there has been no successful therapy for mitochondrial diseases, any progress showing potential of success in mitochondrial medicine should be encouraged, even if we have not fully

understood how it works and still face some challenges. Many works have reported that mitochondrial dysfunction can be alleviated by mitochondrial transplantation depending on various routes of mitochondrial delivery. Simultaneously, mitochondrial gene therapy such as mitochondrial gene transfer and mitochondrial gene editing technologies specifically targeting mitochondrial genes is emerging as another potential approach to treat mitochondrial diseases without multiple injections as mitochondrial transplantation for the long-term therapeutic effect[72−76]. Despite several challenges, mitochondrial transplantation and other novel technologies such as mitochondrial gene therapy open a new avenue for treatment strategies of mitochondrial diseases in which conventional therapies have proved unsuccessful.

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Author contributions

Tianguang Zhang and Chaoyu Miao conceptualized the manuscript. Tianguang Zhang wrote the manuscript and produced the figures. Chaoyu Miao revised the manuscript. The authors read and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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