Expert Opinion

BioTechniques

Considerations for developing mitochondrial transplantation techniques for individualized medicine

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BioTechniques 76: 125–134 (April 2024) 10.2144/btn-2023-0072

First draft submitted: 9 August 2023; Accepted for publication: 6 February 2024; Published online: 29 February 2024

TWEETABLE ABSTRACT

Mitochondrial transplantation has been used to treat various diseases associated with mitochondrial dysfunction. Here, we highlight the considerations in quality control mechanisms that should be considered in the context of mitochondrial transplantation.

KEYWORDS:

individualized medicine • mitochondria diseases • mitochondria transfer • mitochondria transplantation

Mitochondria serve numerous biochemical functions for maintaining cellular homeostasis, such as modifying their structure in response to external stimuli and energetic demand, maintaining a distinct pool of mitochondrial DNA (mtDNA), engaging in intracellular signaling to regulate apoptosis and autophagy and establishing organelle contact sites to facilitate the transfer of substrates between organelles [1,2]. With so many diverse functions, it is no surprise that mitochondrial dysfunction is associated with numerous age-related pathologies, such as through producing reactive oxygen species byproducts (ROS), including cardiovascular, Parkinson's, Alzheimer's and Huntington's diseases, which highlight the importance of developing mitochondria-targeted therapies [3]. In recent years, researchers have become increasingly interested in cell-to-cell mitochondrial transplantation as a strategy to restore normal mitochondrial function and cellular homeostasis [4].

The concept of mitochondrial transplantation was conceived over 30 years ago, although advances in our understanding of cellular biology, organellar dynamics and molecular techniques have led to recent and promising clinical applications. To briefly summarize, mitochondrial transplantation was developed by Clark and Shay in 1982 [5], microinjection, a form of mitochondria transplantation, was further performed preclinically by King and Attardi in 1988 [6], and shown viable in a range of tissues by McCully *et al.* in the past two decades [7,8]. In 2017, mitochondrial transplantation was successfully utilized in Boston Children's Hospital to restore injured myocardium and today there are clinical trials to elucidate the efficacy of mitochondrial transplantation [9] in therapeutic cases including cerebral ischemia [10].

Since then, mitochondrial transplantation has continued to be evaluated and utilized across a variety of disease states [11]. For example, exogenous mitochondria in male mice in a model of fatty liver showed reductions in lipid accumulation and oxidative stress concomitant with restored ATP generation [12]. Yet, other disease states show more conflicting results. In a rat model of experimental spinal cord injury, injection of exogenous mitochondria into the mediolateral gray matter recovered mitochondrial respiration, although did not abrogate impaired motor and sensory functions [13]. Yu and colleagues showed that not only were mitochondria important in arresting malignant subcutaneous and metastatic melanoma, but that female animals induced a more persuasive mitochondria-nuclear communication and were more efficient in anti-tumor activity compared with male mitochondria [14].

Many neurological diseases, among other disease states, are marked by dysfunction of the mitochondria [15,16]. For example, Huntington's disease, a neurodegenerative disorder, is marked by altered mitochondrial protein distribution, impaired mitochondrial ultrastructure [17] and abnormal mitochondrial dynamics and biogenesis [18]. It has been shown that inhibition of excessive mitochondrial fission can restore mitochondrial function, thus slowing the progression of Huntington's disease [19]. Currently, many therapies for Huntington's disease, like other neurodegenerative diseases, are aimed at stem cell-based therapy, such as proactive transplantation of human neural stem cells [20]. Yet, mitochondria transplantation offers a new research avenue that specifically delivers mitochondria to affected neurons, thus allowing the targeting of mitochondrial dysfunction to be individualized to the disease states. Indeed, in a murine model of Parkinson's disease, mitochondrial replacement therapy impairs disease progression by improving electron transport chain activity and reducing oxidative stress and cell death [21]. This suggests the study of mitochondria transplantation as a key clinical avenue, both to

overcome some traditional limitations of stem cell-based therapies (e.g., immune rejection) as well as to complement existing therapies to enhance overall treatment efficacy.

Despite the rise in clinical applications, mitochondrial transplantation is currently limited in use by a lack of robust delivery protocols [1]. Furthermore, our current understanding of quality control mechanisms that govern the mitochondrial structure, oxidative phosphorylation (OxPhos) capacity, protein synthesis and distribution, mtDNA integrity and ROS generation is limited and requires further study. Here, we discuss these factors within the framework of currently available mitochondrial transplantation techniques. We highlight aspects of mitochondria structure and function, including proteins associated with cellular metabolism and oxidative stress, which remain poorly understood in the context of mitochondrial transplantation.

Mitochondrial transplantation techniques to consider

Mitochondrial transplantation generally refers to the isolation and subsequent successful transfer of mitochondria into host cells. There are various methods of transplanting mitochondria, as previously reviewed [22–26]. While we will principally focus on the challenges associated with these methods and unknown factors in the context therein, this section will briefly discuss some of the main or novel methods.

Microinjections

As one of the original methods of mitochondrial transplantation, microinjection functions through up-taking mitochondria, from healthy donor cells or tissues, using a micropipette and injecting the solution of host mitochondria into the cytoplasm of a target cell [6,8,27]. In preparing mitochondria for this, typically differential centrifugation must be performed while maintaining mitochondrial membrane integrity [28]. Microinjection-mediated mitochondrial transplantations have since been optimized for various tissue systems [7], as previously reviewed [25]. Microinjection is one of the only methods for *in vivo* transplantation, and certainly the most developed and well-studied way of mitochondrial transplantation for tissue systems. While microinjection has mainly been done with allogenic or autologous transplantation, it has been marginally effective with xenografts, with sub-1% efficacy (or success rate) [15,29]. Beyond this, while there are multiple ways to inject mitochondria in tissue [7,8], *in situ* microinjection, which has many of the same underlying factors as *in vitro* microinjections, has been shown to be the most effective [30].

Given microinjection is the principal technique that may be employed for *in vivo* techniques, it has the greatest potential to be developed for individualized medicine. Principally, this microinjection technique has been used to replace damaged mitochondria with allogeneic and xenogeneic-sourced mitochondria in a rat model of Parkinson's disease restored mitochondrial dynamics and improved bioenergetics, resulting in greater capability of neurite outgrowth and long-term improvements in locomotive function [31]. Beyond this, in Alzheimer's disease-like pathology in olfactory bulbectomized mice, *in vivo* techniques of microinjection have been utilized to deliver allogenic mitochondria to the brain, where they localized to neurons and attenuated cognitive impairments [32]. It has also been successful in a wide variety of cases including restoring fertility and improving egg quality [33]. Beyond this, it has been successful in protecting the heart from ischemia-reperfusion injury [34].

Microinjection techniques do have important limitations, namely the high risk of mitochondrial damage and relatively low efficiency (due to resource burden, time effort and skill required) and efficacy (due to limited uptake), as previously reviewed [15,29]. Alongside this, given the size of the needles used for microinjection, techniques often target oocytes or single-cell embryos, which are larger and may then proceed to target recipient cells [35]. Nonetheless, together this demonstrates the potential practicality of microinjection already for individualized medicine and treatments, yet other mitochondrial transplantation techniques should also be considered.

Coincubation & centrifugation

Centrifugation is a method of both isolation and delivery of mitochondria; following differential centrifugation for isolation, delivery of mitochondria is performed through a centrifugal force [36]. This technique is generally highly efficient: recent results, show that autonomous mitochondria were used for centrifugation-based uptake in neural cells with Alzheimer's disease-like pathology, with results showing that mitochondrial transplantation can restore damaged neuronal cells [37]. This protocol makes it particularly useful for *in vitro* use cases, although its exact efficacy in selecting 'high-quality' (see Considerations for Implementation of Methods and Future Technique Development section) mitochondria remains unclear [29]. As a method of delivery, centrifugation is the most readily available and may have the lowest technical skill barrier, yet it is limited to *in vitro* applications with relatively low efficacy [29]. Similarly, coincubation, which is a method of indirect transfer with donor and recipient cells cultured together, simply aims to pass on mitochondrial DNA and genetic information through exclusively *in vitro* models, as another relatively simpler technique [30]. This is a less abrasive technique since it does not include the centrifugation step. However, it has relatively low efficacy ranging from 0 to 17% across 3 days [38] as well as relatively slow speed due to a lack of specificity.

MitoCeption, a recently developed protocol, adds thermic shock and centrifugation steps to improve mitochondria uptake in the receiving cells, thus combining centrifugation and coincubation [39]. Notably, while studies with MitoCeption remain limited due to its recency, they show that this technique can alter the recipient cell's metabolism, indicating this is an effective technique with successful uptake [39]. Furthermore, this method may be even further optimized in efficiency by directly adding mitochondria to centrifuges [36].

This unique method has been validated to transfer mtDNA [39] making it especially of use for studying how different cell types, or disease states, may undergo changes when mtDNA heteroplasmy (see Considerations section). Yet, is also limited to *in vitro* techniques, imitating the therapeutical potential of this technique and making it more practical for basic science studies.

Photothermal nanoblade

This method functions on the basis of using a photothermal nanoblade to cut the plasma membrane of somatic mammalian cells for delivery of the mitochondria, allowing for a limited of number of cells to undergo transplantation per hour [40]. Photothermal nanoblade has moderate efficacy in disease-like states (around 2% into mtDNA eliminated cells) but was originally developed for small populations [30]. While this efficacy is objectively still quite low, given the emerging nature of mitochondria transplantation this technique is still promising. More recently, a biophotonic laser-assisted surgery tool has been developed which may allow for the populations of mitochondria for study to be broadly expanded [41], but further investigation must be done on the large-scale usage of photothermal nanoblade, especially as it pertains to medical techniques given its highly specialized equipment requirements. This is especially true as current use cases seem limited to superficial structures, which may limit scalability.

Magnetomitotransfer

Another technique is using magnetomitotransfer to bind to TOM22 in mitochondria on the basis of magnetism of anti-TOM22 beads, as previously reviewed [30,38]. This magnetism added to coincubation can allow greater specificity, so this technique has very high mitochondrial uptake (78–92% and at a faster speed than coincubation) [38]. However, it can transplant dysfunctional mitochondria (see Considerations section) and can be toxic *in vivo* [29]. While magnetomitotransfer remains an interesting new mechanism, it remains unclear in the few studies which have utilized it how mtDNA is changed and if it may be further optimized to maintain mitochondrial quality [38]. Nonetheless, it is a novel potential technique that should be further investigated, especially in seeing if other magnetism can be utilized in the context of *in vivo* applications.

Peptides & polymers

There are numerous techniques being developed to improve mitochondrial transplantation techniques. Cell-penetrating peptide known as Pep-1 can also be conjugated to mitochondria to increase their uptake rate [29,42], often used alongside microinjection methods [31]. This mechanism functions on the basis of the ability of Pep-1 to act as a chaperone, enacting cellular uptake and release of mitochondria [42]. Notably, Pep-1 was able to restore mitochondrial dynamics proteins and structures in mtDNA mutation states (see 'Consideration' section). Similar to Pep-1, Biocompatible polymers, namely dextran with lipophilic cation triphenylphosphonium, have also risen in prominence as a method to conjugate to mitochondria to improve their uptake [9,43], yet it remains unclear if conjugation of such polymers may affect other aspects of mitochondrial function. Thus, while these are both highly promising potential conjugates, more studies are necessary to access how they may interact with novel proteins developed in disease states such as Alzheimer's and Parkinson's disease, should they develop to the point of *in vivo* studies. Mitochondrial transplantation technology is rapidly evolving with promising clinical applications.

Considerations for isolation

The effectiveness of mitochondrial transfer as a viable therapy is limited by the quality of mitochondria that can be successfully isolated and transferred, therefore highlighting the need for strategies to reliably evaluate mitochondrial fitness and isolate 'fit' mitochondria is important. As discussed, most techniques employ differential centrifugation, which involves two steps of low and high-speed centrifugation to remove debris and concentrate mitochondria, respectively [44]. However, density gradient centrifugation or affinity purification are other techniques that can separate and further purify mitochondria, as extensively discussed [45]. Furthermore, differential filtration techniques have previously been optimized for mitochondrial isolation [44]. However, generally, these techniques' effectiveness varies among cell types, thus specific protocols for respective cell types should be obtained [45,46]. These techniques can further be improved through the adhesion of certain proteins or paramagnetic microbeads [47]. While these are considerations for macro-scale isolation, past reviews have extensively covered considerations for both macro- and micro-scale isolation [48].

Notably, while isolation is typically separate from the injection process, integration may reduce some of the burdens that come with storing mitochondria prior to transplant [24]. Recently, a nanosyringe-mediated FluidFM-based technique facilitated the direct transfer of mitochondrion with high efficacy but limited efficiency [4]. As opposed to traditionally centrifugation-based techniques for isolation of mitochondria before transplantation, this technique combines atomic force microscopy principles with microfluidics to use a microinjection to both extract and inject mitochondria [4]. This technique is unique in having high accuracy in fusion within the greater mitochondrial network, as well as potentially avoiding mtDNA conflicts through the replication of donor mtDNA [4]. Thus, integrated techniques such as this are important potential therapies and will likely continue to be developed.

Considerations for implementation of methods & future technique development

Currently, the successful application of mitochondrial transplantation in a clinical setting has been hampered by various technical and biological issues, including specificity, transplantation rejection, and scalability, which can impair mitochondrial biogenesis and cell sur-

vival [9,22,29]. As part of the transplantation process, mitochondria are removed from their native cellular surroundings and exposed to external factors, including temperature, potentially generating changes in ROS and mitochondrial structure [30]. Still, it is unclear if the success of these methods is dependent on certain mitochondrial conditions [15], thus there is a need for numerous considerations in the application of current techniques and the development of future methods. To address the limitations in the validation of mitochondrial transplantation, it is essential to consider the viability of mitochondria before and after transplantation by testing mitochondria ultrastructure, motility, organelle contacts, protein distribution, mtDNA changes, ROS and other factors. Until these features are elucidated, the applicability of mitochondrial transplantation as a method for individualized medicine may be limited.

Oxidative stress

Oxidative stress, while naturally occurring as a byproduct of respiration over time, can also impair mitochondrial function and should be considered in mitochondrial transplantation. In mitochondria, oxidative stress is regulated by the uncoupling proteins (UCPs), mainly 2 and 3 which may potentially prevent oxidative stress [49,50]. Furthermore, it is essential to consider preserving optimal levels of metabolites that regulate ROS, such as NAD, as well as transcriptional circuitry that modulates mitochondrial biogenesis [51].

Currently, results are conflicting on the causative effect of mitochondrial transplantation on oxidative stress. Studies have shown that mitochondrial transfer can also induce oxidative stress [52], which may also hold true for mitochondrial transplantation and may serve as an indicator of an unsuccessful transfer [24]. However, transplantation can also be used to alleviate oxidative stress in disease states. Notably, recent findings have demonstrated that transplanted mitochondria are able to cross the blood–brain barrier and buffer ROS following status epilepticus [53]. This is consistent with studies focusing on the anti-tumor potential of mitochondrial transplantation, which can result in reduced oxidative stress [54]. Still, during the transplantation process mitochondria are vulnerable to generating oxidative stress. During reimplantation and reperfusion, antioxidants, such as MitoSNO [55], S1QELs [1] and malonates, as well as hypoxia during storage, can be used to reduce ROS and improve transplantation efficacy [24]. However, conversely, other studies have shown that exposure to oxidative stress may enhance mitochondrial uptake [56]. Thus, managing oxidative stress, and determining the positive or negative effects of ROS on mitochondrial transplantation, is an important consideration in determining mitochondrial transplantation viability.

Mitochondrial dynamics

In general, the insertion of exogenous mitochondria is dependent on reestablishing fusion and fission dynamics, which are essential for normal function [29,57]. Past studies have suggested that mitochondrial function may be retained following transplantation [53,58], but how mitochondrial dynamics proteins are altered following transplantation remains poorly understood. Notably, one study has shown that shortly following mitochondrial transplantation there is a rapid uptick in the bioenergetics [59], suggesting that mitochondrial structure or cristae may be optimized to increase ATP production such as through modulation of dynamic proteins. Mitochondrial dynamics are highly relevant in a multitude of disease states, such as Parkinson's Disease, wherein mitochondrial transplantation may have a restorative effect [31], indicating the importance of its consideration in developing individualized medicine techniques. However, is unclear whether the expression of fusion and fission proteins is affected following mitochondria transplantation, which may affect whether normal mitochondrial biodynamics can be re-established. One study has shown that for a short period of time following transplantation, there is an uptick in fusion proteins and a decrease in fission proteins [60], but further studying across a range of transplantation methods is necessary.

Another potential area to explore is if supplementing mitochondrial proteins following transplantation, such as OPA1 and the MICOS complex, which are both needed for mitochondrial cristae architecture [61,62], could restore mitochondrial dynamics. For example, the delivery of these proteins following transplantation may allow for alternative, less invasive pathways of mitochondrial dynamics; however, published research on this topic remains limited. Furthermore, additional proteomic studies are needed to determine the pathways and proteins that appear under certain mitochondrial conditions, as these may affect mitochondria dynamics during transplantation. In particular, the enrichment of certain proteins, such as UCPs, following transplantation may serve as potential indicators of successful transplantation.

MtDNA

Mitochondrial transplantation is limited by our current understanding of whether mitochondrial DNA (mtDNA) can return to normalcy and establish a functioning mitochondrial network following transplantation. If not properly regulated, mitochondrial DNA can serve as a damage-associated molecular pattern, resulting in immune response activation [24], but it remains unclear how mtDNA quality both alters transplant efficiency as well as is affected long-term following transplantation. Recent studies have suggested that mtDNA quality may play a role in the development of age-related diseases and that therapeutic interventions that target mtDNA structural and functional integrity may be effective in preventing or treating these diseases [63].

Notably, additional studies have suggested that transplanted mitochondria can introduce new mtDNA variants [25,39,64], which may have implications in nuclear crosstalk; however, how heteroplasmy affects the efficacy of mitochondrial transplantation continues to be poorly understood [4]. Heteroplasmy may occur both due to genetic variation [65], as well as exposome factors, such as through lifestyle choices, thus serving as potentially analogous to biological aging [66]. Given variable sources of mtDNA heteroplasmy, the

persistence and replication of donor-derived mtDNA and its interaction with recipient mtDNA, in the case of allogenic transfer, are critical factors in understanding the long-term efficacy and viability of mitochondrial transplantation therapies. In particular, a study of ooplasmic transplantation showed that embryos, amniocytes, fetal placenta and cord blood showed sustained mitochondrial DNA heteroplasmy representing, potentially affecting fetal development [67]. Contrastingly, mitochondrial transfer to human oocytes has been shown to improve embryo quality without changing maternal mtDNA sequence [68]. Yet, to our knowledge, few other studies like this have been performed in the context of mitochondrial transplantation.

Specifically, it is unclear how mtDNA copy numbers and quality are regulated during transplantation. Maeda and colleagues recently demonstrated a method to generate mtDNA-replaced somatic cells, which may potentially be recapitulated to abrogate heteroplasmy in patient-derived fibroblasts for mitochondrial transplantation [69]. *Ex vivo* methods of mtDNA repair can rescue only small areas of tissue, underscoring the need for scalable methods for clinical application [22]. Another area of study is whether mtDNA genome editing tools, such as mitoTALEN, be used to alter mitochondrial quality [70] but need further consideration in the long-term viability of mitochondria following transplantation. In addition, it is ambiguous if mtDNA similarity is necessary for transplantation, which may require transplants between individuals of similar ancestry, and how this could affect future treatments. Interestingly, past studies have shown that the endogenous innate immune-activating molecules of injected mitochondria can result in increased allograft rejection, suggesting that consideration of how to modulate the immune response in response to allogenic mitochondria is an area for further study [71].

Mitochondrial 3D structure

As reviewed, past literature on mitochondrial transplantation focused on increasing the total mass of the mitochondria [9,22,24]; however, mitochondrial function goes far beyond this. The role of mitochondrial structure, cristae shape and organization are important determinants of mitochondrial function [72,73]. While cristae provide surface area for the electron transport chain, mitochondrial structure can also dynamically respond in response to metabolic needs [73]. For example, disruption of cristae architecture can lead to mitochondrial dysfunction and disease [72]. The organization and quality control of mitochondrial cristae is an area that has received increased attention in recent years, and its structure, which is linked to its function, may be studied through transmission electron microscopy quantification techniques [74,75], facilitated by proper fixation protocols [76]. However, while the 2D structure of mitochondria is important to study [75], equally so, the 3D structure of mitochondria following transplantation must be studied.

Currently, mitochondria have eight known structural phenotypes: nanotunnels, donut-shaped, branched, large volume, small volume, elongated, compact and mega mitochondria [73,77]. Many of these methods can be imaged with proper serial block face scanning electron microscopy [78,79] or focused-ion beam scanning electron microscopy [80] techniques. While the exact conditions and mechanisms by which mitochondria form these phenotypes are poorly understood, they are thought to be associated with metabolic demand triggering the formation of these unique structures through fusion and fission [73]. However, it is also possible that specific types of fission and fusion, or distribution of their respective proteins, aid in determining a specific structural phenotype [81]. It remains unclear if mitochondrial activity could be modulated through the selection of specific mitochondrial phenotypes. In particular, are certain mitochondrial phenotypes more energetically favorable for transplantation than others and can these phenotypes be modulated? For example, nanotunnels in mitochondria may impair the efficacy of mitochondrial transplantation. Alternatively, it is also possible that the relative ratio of mitochondrial phenotypes must be maintained across transplantation. Thus, beyond looking at only ultrastructure prior to transplantation, it is also important to determine if mitochondrial transplantation is more favorable to certain phenotypes, or if mitochondria modify their structure following transplantation. Together, it is clear that the mitochondrial 3D ultrastructure [78], including branching and the wider mitochondrial connectome, needs further consideration to determine the efficacy of transplantation as a therapy.

Mitochondrial contact sites

Another structural consideration is how mitochondria interact with various organelles through the formation of physical contacts following transplantation. The most well-studied form of organelle-organelle contact sites are mitochondria-endoplasmic reticulum (ER) contact sites (MERCs), which are involved in steroidogenesis, signaling pathways, and Ca^{2+} signaling [82]. It is unclear if the viability of transplanted mitochondria can be improved by exploiting close contact with other organelles. However, mitochondria can also form contact sites with other organelles, including machinery involved in the autophagic process [2]. Given that contact sites need tight junctions of under 50 nm, localizing transplanted mitochondria near specific organelles, such as ER and lipid droplets, may improve long-term viability through increasing metabolism [83]. Notably, recent cardiac therapies have utilized artificial tethers to facilitate MERC formation [84], so greater research into whether these transgene tethers may be employed alongside mitochondrial transplantation can be promising. The necessary organelle contact sites for improved cell viability may also be in a tissue-dependent manner, as lipid droplet contact sites are thought to be more important in the fatty-acid synthesis [83], while ER contact sites may have different roles. Thus, depending on tissue function, certain organelle contact sites may be necessary for reestablishing mitochondrial networks following transplantation.

Tissue-dependent responses

A final area of consideration is how mitochondrial transplantation differs across different tissues. Mitochondrial function and structure vary across different tissues; for example, while cardiac tissue displays more spherical mitochondria, skeletal muscle displays branched

tissue in youth which may be fundamental to the tissue's function [62,73,85]. Similarly, while brown adipose tissue mitochondria become larger across aging [86], this is not necessarily the case in many other tissue types [85]. Therefore, quality control mechanisms may also differ [73]. Notably, tissues respond differently to aging and pathologies [87]. As reviewed, many transplantation techniques have been unable to achieve tissue and cell specificity [9]. Mitochondria are transferred in disease states in many different tissue types to restore certain cell types, suggesting that intercellular mitochondrial transplantation is viable [88].

The import of localized versus systemic delivery cannot be neglected in considering tissue dependency. For diseases that affect multiple organ systems, systemic delivery might be more effective. Notably, Capelluto and colleagues demonstrated that transplantation of mitochondria from one cell lineage to a different one (i.e., lineage-mismatched) can effectively restore the normal protein composition in cells lacking their own mitochondria [89]. This finding suggests the ability to utilize lineage-mismatched mitochondria for systemic delivery, yet these findings also showed that nonmitochondria-depleted recipient cells do not have altered proteomics following transplantation if enforced depletion does not occur [89]. Together, this suggests that while systemic delivery is plausible, it is reliant on the clearance of mitochondria in targeted areas.

Targeted delivery may provide higher efficacy of mitochondria transfer therapies to the affected area. Lin *et al.*, studying mitochondrial transplantation in traumatic spinal cord injury in rats, found that transplanted mitochondria were detectable in the injured spinal cord up to 28 days and led to improved recovery of functions [90]. Furthermore, Sun and colleagues have developed a polypeptide, CSTSMLKAC that binds triphenylphosphonium cations which aids in mitochondrial translocation to myocardial ischemic therapy [91]. This suggests that localized delivery of mitochondria is possible for specific delivery of mitochondria to certain disease states. However, it is unclear if mitochondrial transplantation efficacy differs between tissues, requiring tissue-dependent mechanisms, and if certain cell types within a tissue are more receptive to transplantation. For example, if one were to transplant hyperbranched mitochondria to cardiac tissue, which does not typically have this type of mitochondrial shape, would transplantation fail? Therefore, it is important to investigate the tissue-specific differences in mitochondrial transplantation and determine the most effective approach for different tissues and cell types.

Mitochondrial horizontal transfer

While not specifically related to mitochondrial transplantation, a better mechanistic understanding of mitochondrial horizontal transfer may aid in the optimization of mitochondrial transplantation techniques. Naturally, cells often seek to do a similar process to mitochondrial transplantation through horizontal cell-to-cell mitochondrial transfer. Notably, mitochondria can travel between neighboring cells through nanotubes or microvesicles, suggesting that mitochondria transplantation is not limited to artificial methods [15,92]. Horizontal transfer *in vitro* and *in vivo* has been reported through microvesicles or tunneling nanotubes to deliver 'healthy' mitochondria from mesenchymal stem cells [15,30]. Endogenous mitochondria transfer is important for maintaining tumor microenvironments, stress response, and metabolism [15]. However, conversely, these similar mechanisms can also lead to dysfunctional mitochondrial transfer, promoting reactive oxygen species and resulting cancer proliferation [52], suggesting pluralistic mechanisms through which mitochondrial transfer may act. Similarly, while extracellular vesicles may be involved in the transport of mtDNA between cells [30,93], it is unclear if there is a dependence on EVs to support mitochondria transfer. Another potential way mitochondria are transferred is through intercellular junctions mediated by connexins [15,94]. As previously reviewed [95], mitochondria may also house connexins, yet it remains unclear if mitochondrial connexins such as connexin 43 can be used to modulate intercellular-junction-based mitochondrial transfer. These mechanistic insights are important to consider, as it remains unclear if simply restoring healthy mitochondria in the surrounding environment will result in horizontal mitochondrial transfer, providing new avenues for mitochondrial transplantation.

Future outlook

Given these considerations, additional studies are needed to improve our understanding of the rapidly advancing mitochondrial transplantation technology and to develop new techniques for individualized medicine. Past reviews have focused on the applicability of mitochondrial transplantation as a cardiovascular therapy [23], highlighting the need to consider this approach in different tissues. For example, in brains, Parkinson's disease can be slowed by decreasing dopaminergic neuron loss using mitochondrial transplantation [96]. The applicability of mitochondrial transplantation to skeletal muscle, kidney tissue, liver tissue, and other forms of mitochondria requires further study, which will be relevant in aiming to deliver mitochondria in specific disease states as a therapy.

Importantly, future studies are necessary to improve mitochondrial uptake. A major issue is optimizing and investigating how isolated mitochondria can be stored [9,22]. Currently, rapid transplant of mitochondria is required as mitochondria are less viable within a few hours [9,22]. New techniques, such as peptide labeling [31] may be used to improve stability. Furthermore, mitochondrial transplantation might have an immunomodulatory effect, which can be positive in some states [97], but conversely can result in an immune-response targeting transferred mitochondria [98]. Autologous mitochondria may be able to reduce inflammatory and immune responses [29], but not completely. However, it may be further possible to reduce immune responses to allogenic mitochondria by selecting specific proteins or structures. Furthermore, monitoring mitochondrial uptake is still limited, and improving our ability to accurately monitor mitochondria uptake may allow better assessment of its improvement. Studies have begun to overcome this limitation through labeling with turbo-green fluorescent protein [13] as well as dyes such as MitoTracker or turbo-green fluorescent protein to verify mitochondrial activity [13,15],

although these can cause dye cytotoxicity. This may be able to be alleviated through new techniques, which can rely on qPCR to measure relative mtDNA heteroplasmy to show mitochondrial uptake over time [56], but these techniques are still developing.

Beyond tissue dependency, mitochondrial transplantation techniques still must be studied and optimized across a range of disease types. For example, in the context of neurological diseases, one of the major limiting factors to mitochondrial transplantation is the blood–brain barrier. Several therapies and techniques are focused on overcoming this limitation. One study has demonstrated that transplanted mitochondria, following status epilepticus, are able to cross the blood–brain barrier [53]. Another study has shown that exogenous mitochondrial treatment improved cellular respiratory control in neuronal and vascular endothelial cell functions post-traumatic brain injury and even reduced blood–brain barrier leakage [99]. Furthermore, inventive strategies to circumvent the blood–brain barrier have been shown to be effective. For example, in the treatment Parkinson's disease, infusion of allogeneic mitochondria via intranasal mitochondrial delivery, using either Pep-1-conjugated or unconjugated mitochondria, showed significant neurological improvement and dopaminergic neuron migration [100]. Similarly, ultrasound activation of microbubbles have been shown to open the blood–brain barrier, thus improving mitochondrial transplantation efficacy, with hemorrhagic complications [101]. Together, these studies demonstrate that many tissue systems and disease states may have unique barriers which must continue to be studied for mechanisms to overcome them, in the context of mitochondrial transplantation.

In addition, alternative sources of mitochondria should also be explored. For example, a recent study observed that the placenta may be a viable source of mitochondria for transplantation [27]. Intrauterine placenta-derived mitochondrial transplantation can be a potential therapy for endometrial injury by repairing mitochondria [102]. This highlights the applicability of mitochondrial transplantation beyond commonly studied tissue types. Pertinently, it is also possible that extracellular environments may alter mitochondria populations in nearby cells [22]. Similarly, it is unclear if mitochondria can be delivered to nearby healthy-cellular populations to increase biological mitochondria transfer to unhealthy populations. While *De novo* synthesis of artificial mitochondria is also a possibility [23,88,102], these same underlying questions regarding how to optimize mtDNA, mitochondrial structure, and other factors, beyond only function, to optimize their viability remain.

Finally, as research continues to demonstrate the potential therapeutic applicability of this technology, there is a need to consider universal access in providing therapy. Currently, the United Kingdom is one of the only countries to have regulated mitochondrial donation, albeit in the context of mitochondrial diseases and through maternal spindle transfer and pronuclear transfer (see https://www.hfea.gov.uk/treatments/embryo-testing-and-treatments-for-disease/mitochondrial-donation-treatment/). Given the cutting-edge nature of mitochondrial transplantation, few countries have critically evaluated it in a clinical setting. While mitochondria transplantation may have a burden in costs through hospital stays, post-transplantation monitoring, and any supplementary treatments required, the ability for autologous transplantation may reduce the current burdens of waitlists currently associated with allogeneic stem cell transplantations [103]. While it will be a considerable amount of time before mitochondrial transplantation is used clinically, the translational aspects of ensuring equitable access to such potentially powerful therapies should be considered alongside its development.

Conclusion

Importantly, the study of mitochondria is not isolated to function and dysfunction. The regulation of mitochondrial dynamics, expression, networks, energetics and diseases continue to be poorly understood [1]. Understanding the role of mitochondria in pathogenesis is vital for the development of effective therapeutic options. A greater understanding of key aspects of mitochondrial regulators is especially relevant. Currently, mitochondrial transplantation is being tested *in vivo*, but the ways it may affect mitochondrial quality remain lacking. Future experiments may determine if modulating mtDNA, proteins, and structure may avoid having the body reject allogenic mitochondria. With so many unanswered questions, it is vital to continue furthering our understanding of mitochondrial modulators and pluralistic mechanisms of quality control to further develop mitochondrial transplantation technology, increase the efficacy of techniques, and ultimately improve its viability as a potential form of individualized medicine.

Author contributions

K Neikirk: literature review, writing – original draft preparation, writing – review and editing. DC Stephens: literature review, writing – original draft preparation, writing – review and editing. AG Marshall: literature review, writing - review and editing. JA Gaddy: literature review, writing – review and editing. SM Damo: literature review, writing – review and editing. A Hinton Jr: funding, conceptualization, supervision, writing – review and editing.

Financial disclosure

The UNCF/Bristol-Myers Squibb EE. Just Faculty Fund, Career Award at the Scientific Interface (CASI Award) from Burroughs Welcome Fund (BWF) ID no. 1021868.01, BWF Ad-hoc Award, NIH Small Research Pilot Subaward to 5R25HL106365-12 from the National Institutes of Health PRIDE Program, DK020593, Vanderbilt Diabetes and Research Training Center for DRTC, CZI Science Diversity Leadership grant number 2022-253529 from the Chan Zuckerberg Initiative DAF, an advised fund of Silicon Valley Community Foundation (to A Hinton Jr). NSF EES2112556, NSF EES1817282, NSF MCB1955975 and CZI Science Diversity Leadership grant no. 2022-253614 from the Chan Zuckerberg Initiative DAF, an advised fund of Silicon Valley Community Foundation (to SM Damo). The funder had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors have no other relevant affiliations

or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, stock ownership or options and expert testimony.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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