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Review Article

Research Progress on Mitochondrial Replacement Therapies in Reproductive Medicine

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Abstract

Mitochondria have been proposed as important factors regulating female reproductive processes, which have a distinct impact on oocyte maturation, fertilization and early embryo development. Mitochondria dysfunction is implicated in disease and in age-related infertility. Therefore, new technologies have opened up new possibilities of mitochondrial replacement/transplantation therapies in oocytes or zygotes, which could prevent the transmission of mutant mitochondrial DNA (mtDNA) to offspring and improve the quality of oocytes of older women. In this review, we attempt to explore aspects of the distinctive contribution of mitochondria to reproductive processes, and discuss current and emerging clinical implications.

Keywords: Mitochondria; Mitochondrial replacement therapy; Mitochondrial transfer therapy; Infertility; Oocyte quality

Introduction

Mitochondria are essential small organelles exclusively of maternal inheritance and are involved in generating cellular energy, mediating cellular apoptosis and regulating gene expression (Figure1) [1-5]. The mature human oocyte contains more mitochondrial and mitochondrial DNA (mtDNA) than other cell types, and several hypotheses implicate mitochondria as crucial factors regulating reproductive capacity [6].

Infertility has become a growing problem worldwide, and decreased oocyte quality is the main factor culminating in the age-related deterioration of reproductive capacity [7]. Mitochondria play key roles in oocyte functions and they are critical indicators of oocyte quality. Mitochondrial functions are important for the formation of meiotic spindles and for maintenance of the MII spindle before fertilization. All of the complex processes the oocyte goes through prior to ovulation and fertilization require energy, which is derived mainly from Adenosine Triphosphate (ATP) via mitochondrial OXPHOS [8]. Early embryo development and implantation rate have been correlated with mitochondrial function and activity [9]. It has been shown that insufficient ATP production during the oogenesis and embryogenesis will result in aneuploidy, a condition in which chromosomal segregation errors are frequently encountered [10]. Moreover, clinical data suggest that higher ATP content from human oocytes and embryos has been associated with better reproductive results among infertile patients [11]. Therefore, various attempts have been made to restore oocyte quality by improving mitochondrial function. The past decade has seen enormous advances in potential therapies to restore oocyte quality and transfer of mitochondria from cells with mitochondrial integrity into mitochondria-impaired oocytes [12]. Novel technologies have opened up new possibilities of mitochondrial replacement/transplantation therapies, which will increase the success rates for *In Vitro* Fertilization (IVF) of oocytes from women with poor oocyte quality. In this mini-review, we will present recent evidence to discuss: 1) Mitochondrial dysfunction in quality-compromised oocytes; and 2) Possibilities to overcome mitochondrial dysfunction in oocytes to improve IVF success rates.

The Role of Mitochondria in Oocyte and Early Embryo

Mitochondrial dynamics and activation in oocyte maturation, fertilization and embryo development

Mitochondrial proliferation and activation in pre-implantation are crucial for the subsequent development [13]. In general, mitochondria in embryos are mainly derived from oocytes. There is accumulating evidence that the quality or quantity changes of mitochondria in oocytes would have profound effects on early embryo development. According to bottleneck theory, mtDNA pool could be renovated and decontaminated by a drastic reduction in the primordial germ cells followed by a great amplification during oogenesis [10]. Mitochondrial number in mature oocyte has been increased 1000 times from only few dozens to more than 100 thousand ones when compared to primary follicles [14,15]. Then, mitochondria are averagely distributed to each blastomere during embryonic development, but the total number is relatively stable before the blastocyst stage [16]. Likewise, mitochondrial activity is

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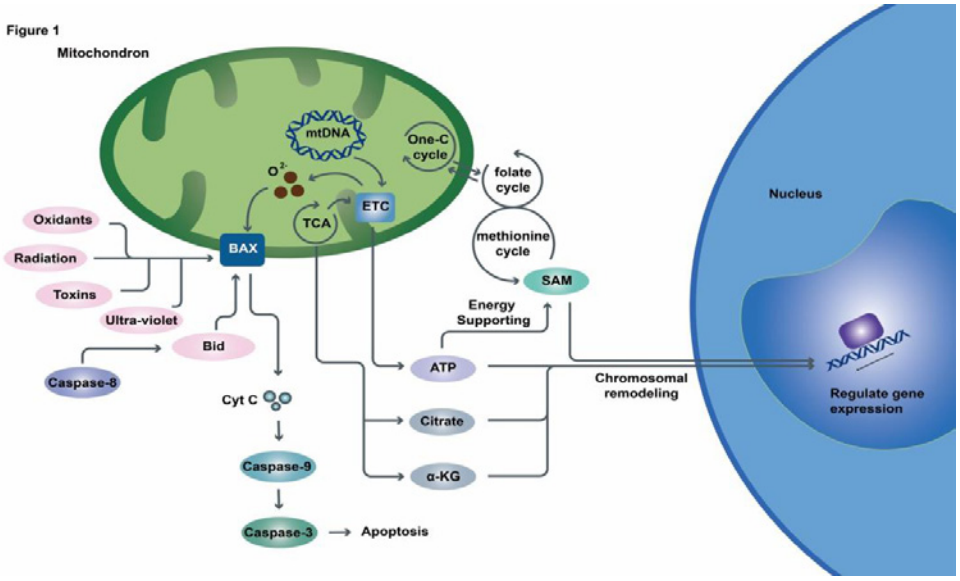

Figure 1: The role of mitochondria in metabolism, apoptosis and gene expression. For caspase activation by mitochondria, multiple stimuli (such as oxidants, radiation, toxins or ultra-violet) can modify permeability of mitochondrial membrane by activating Bax then releasing Cyt C from mitochondria into the cytosol. Thereby, Cyt C triggers caspase-9 and initiates the proteolytic cascade that culminates in apoptosis. Simultaneously, caspase-8 can also activate Bax by slicing Bid, which results in the similar apoptosis pathway. Additionally, mitochondria could provide enough energy for cell by synthesize ATP via ETC. Mitochondrial metabolites of TCA cycle (such as ATP, α-KG and citrate) could also regulate gene expression via remodeling chromatin. ATP synthesis is dependent on mitochondria oxidative phosphorylation driven by the ETC and ATP synthase. SAM is one of metabolites of methionine and folate cycles that supported by mitochondrial one-carbon cycle. While SAM synthesis requires ATP to support necessary energy for chromatin modulating and then binding to genes specially. α-KG, α-Ketoglutaric acid; TCA, Tricarboxylic acid cycle; ETC, electron transport chain; SAM, S-Adenosyl methionine; Cyt C, cytochrome C.

also relatively stable before the blastocyst stage. Mitochondria in mammalian oocytes are transcriptionally and bioenergetically silent and this functional state appears to be evolutionarily conserved [17], especially in immature eggs, which have slow ATP production [18]. This quiescent state is believed to be important to keep the number of mtDNA mutations to minimum, due to these mutations will then be passed down to the embryo [19]. Therefore, the energy required for development is mainly provided by surrounding granule cells and cumulus cell [20,21]. It has been reported that mitochondrial dysfunction may cause spindle and chromosomal abnormalities with the development of follicles [22]. Given the mechanism of self-protection, mitochondria remain keeping relatively low activity until developing to the blastocyst stage in order to reduce superfluous oxygen free radicals that may cause deleterious damage to cells [23]. Previous studies have also demonstrated that overfull replication of mtDNA could reduce early embryo activity and implantation potential [24]. When the embryo develops to the blastocyst stage, mitochondria in the embryo have become slender, the structure of ridges are intact, and the mtDNA is largely replicated, which indicate that mitochondria have completed the transition from quiescence to activation [25,26]. Thus, it has been suggested that mitochondrial dynamics are involved in the mechanisms of superfluous low-active mitochondria in oocyte to sustain early embryonic development where mitochondrial replication is restrained in order to protect embryo from oxidative damages.

Mitochondrial dysfunction and oocyte quality

Oocyte quality is great important for fertilization and development into viable offspring. Quality-compromised oocytes are correlated with infertility, developmental disorders, reduced blastocyst cell number and embryo losses, however, the mechanisms underlying these effects are not yet well understood. Oocyte quality is achieved during the maturation process. Mitochondria are critically important for oocyte maturation. Many of maturation defects may have been correlated with mitochondrial dysfunction [27] due to insufficient ATP and several others. Thus, mitochondrial dysfunction in oocyte is believed to be a critical factor for older infertility patients with poor oocyte development [28-30].

In oocytes, mtDNA copy number and mitochondrial activity are important for oocyte quality. Several studies reported that the mtDNA copy number in the arrested oocyte was reduced significantly when compared to that in normal oocytes [31,32]. Previous reports have shown that mtDNA copy number of oocytes in older women (age >36 years old) down sharply, but the frequency of mutation and depletion aggregate intensely [33,34]. It suggests that mitochondria are low-count and dysfunction in these patients. Mitochondria are essential for cytoplasmic maturation to contribute ATP which is needed for critical cytoplasmic and cellular functions [35]. Declining of mitochondria would reduce the ATP production, while oocytes require enough mitochondria-generated ATP to offset the energetic shortage as a result of high-energy consumption progress such as meiosis, cleavage and fertilization. Beyond energy deficiency, the failure to produce enough ATP would also have destructive consequences on chromosome assembling and further to disturb normal gene expression and homeostasis. Furthermore, mitochondrial dysfunction would induce the production of other mitochondria-related metabolites such as citrate and α-KG, which would have an alteration on epigenetics and then affect oocyte quality. Mitochondria-deficient mice with loss of mitochondrial

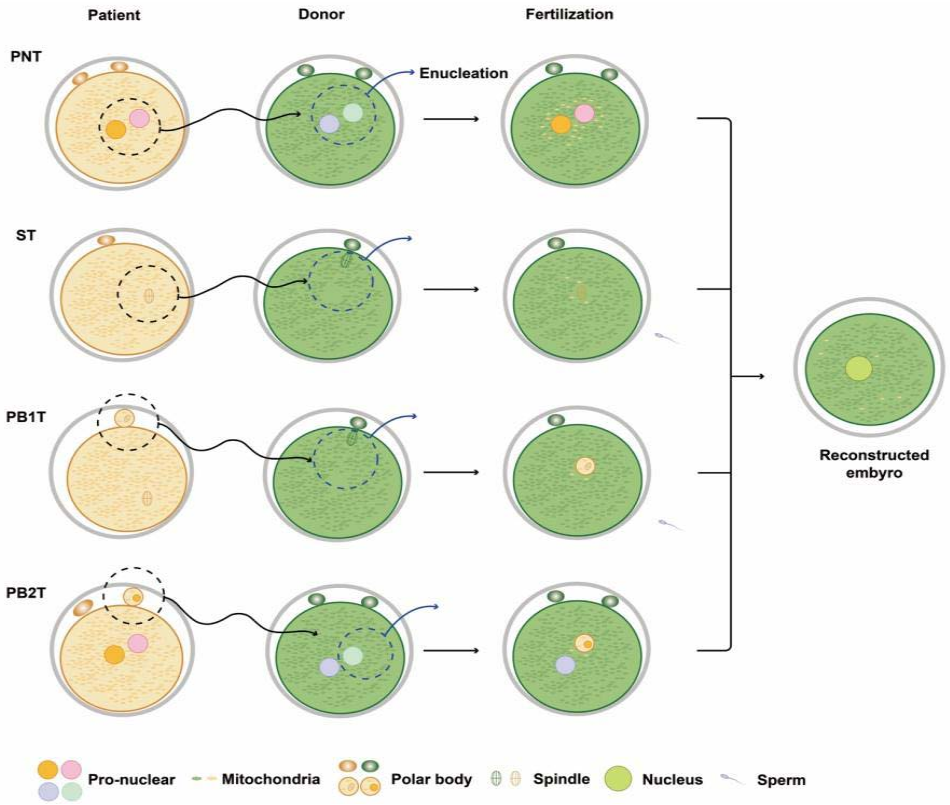

Figure 2: Schematic diagram of Mitochondrial Replacement Therapies (MRTs). The procedures of Pronuclear Transfer (PNT), Spindle Transfer (ST), the first/second Polar Body Transfer (PB1T/PB2T) are showed, respectively. Briefly, the karyoplast was isolated from the patient oocyte or zygote, and then transferred into the enucleated recipient oocyte or zygote.

peptidase Clpp is infertile completely and has no mature oocyte [36]. Other researches also identified that mitochondrial deficiency would accumulate mutational mtDNA and produce excessive oxidative respiration, thus leading to metabolic disturbance and poor oocyte quality [37]. Hence, mitochondria-dependent pathway disorder would be a primary contributor to cause follicular atresia. It is believed that mitochondrion is a core factor in oocyte development not only for supplying energy but also for involving in multiple signaling pathway implicated in gene expression [38].

Possibilities to overcome mitochondrial dysfunction in oocytes to improve IVF success rates

One of the most challenging problem for achieving successful IVF and embryo development is the poor quality of mitochondria in patients with advanced reproductive ages and with various other metabolic disorders. This limitation has stimulated a number of different approaches to overcome the defects. As mitochondrial dysfunction negatively affects oocyte quality, an increase in mitochondrial number and/or function improvement of mitochondria could potentially help to improve fertility.

Techniques for mitochondrial replacement therapy (MRT)

The mtDNA mutations are a relatively common cause of mitochondria disorders, there is currently no cure for these disorders. Therefore, a challenge in most cytoplasmic transfer techniques is to completely remove mtDNA from the patients. In recent years, MRT has been developed to eliminate transmission of mtDNA to offspring and improve the quality of oocytes of older women [39]. MRT is a process that the nucleus is moved from oocyte or zygote with abnormal mtDNA then transferred into donor oocyte or zygote containing healthy mitochondria. MRT could prevent transmission of mtDNA disease to offspring [40]. MRT can also increase mitochondrial function and energy supply in aged oocyte. Generally, there are several novel approaches for circumventing mtDNA-based transmission that implicate germline gene therapy including Germinal Vesicle Transfer (GVT), Pronuclear Transfer (PNT), Spindle Transfer (ST) and Polar Body Transfer (PBT) (Figure 2) [41,42].

Germinal vesicle transfer (GVT)

GVT has represented useful a tool for exploring the interaction between the nucleus and cytoplasm in the oocyte maturation process in mammals [43]. Furthermore, it has previously been reported that the invasive nuclear handling does not compromise fertilization or subsequent embryo development [12]. To some extent, efficiency might be due to recipient cytoplast competence. Therefore, using the human model, transferred GV can progress to the MII stage but follows cytoplasmic dominance for species-specific maturation dynamics and rates. In early previous, Zhang et al. [44], found that four out of seven human oocytes reconstructed from GV of old oocytes (women age > 38 years) with cytoplasm of young oocytes (women age < 31 years) display normal second meiotic chromosome complement. What's more, it has been reported that GVT could have a positive impact on oocyte maturation, fertilization, and embryo cleavage, maintaining normal ploidy [45]. However, GVT is defective. This method would

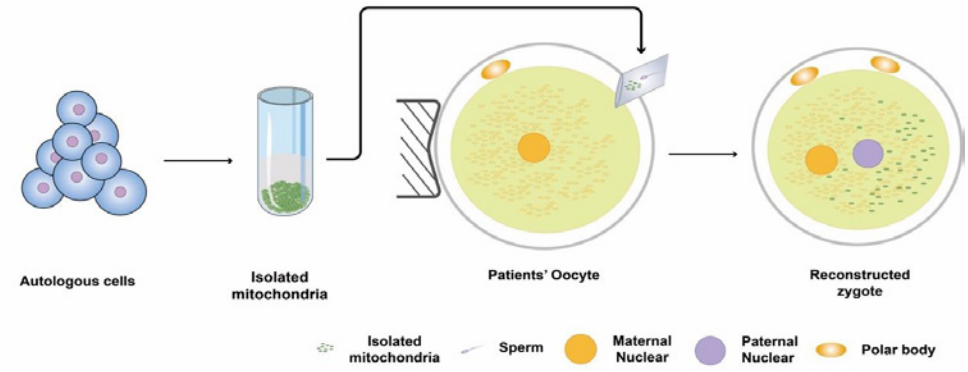

Figure 3: Technological process of mitochondrial transfer therapy (AUGMENT). Mitochondria were isolated from autologous cells germlines such as granular and cumulus cells, oogonial stem cells or mesenchymal stem cells from diverse tissues, and injected into oocyte with a sperm by ICSI to form a reconstructed zygote.

carry multiple impurity such as mRNAs, proteins, mitochondria or other organelles to new embryos and have comparatively high risks of chromosomal abnormalities and pregnancy failure [46]. Thus far, it is hard to evaluate the inherited risk of two disparate mitochondria coexists in the same embryo and this heterogeneity would not be good things to generations.

Pronuclear transfer (PNT)

The zygote stage in mammals is characterized by the presence of two Pronucleus (PNs), each containing a haploid chromosomal complement of nuclear DNA [47]. The method of PNT involves removing two PNs after treatment with cytoskeletal inhibitors such as cytochalasin B or nocodazole, and then transferring them to a recipient zygote that has had its own PN removed. Earlier in 2005, experiment on mice have shown that PNT is feasible to replace deficient mitochondria [48]. In 2010, the feasibility of PNT in the human was reported by Craven *et al.*, [47] using abnormal zygotes with either one or more than two PNs. They found the average level of mtDNA carryover is less than 2%, with many of the embryos containing no detectable donor mtDNA. More recently, Louise *et al.* have developed an alternative approach based on transplanting pronuclei shortly after completion of meiosis rather than shortly before the first mitotic division [49]. In their study, mtDNA carryover was reduced to < 2% in 79% of PNT blastocysts after optimization. Nevertheless, PNT require fertilizing both the donor and the recipient oocytes, which results in discarding half of the embryos during manipulation. This practice would generate amounts of obsolete embryos which may largely induce disputations on morals. Furthermore, related evidences indicated that mitochondrial carryout from PNT could increase rapidly after fertilization and induce high mitochondrial heteroplasmy level (ranging from 5%-44%) after birth in mice [50]. Interestingly, Wu et al., [42]. recently found that Pre-Pronuclear Transferring (PPNT) is more suitable than PNT. Pre-Pronuclear (PPN) can exist 3.5-6 h in human after fertilization, and thereafter, it will be enclosed by the pronuclear envelope and forms the female pronuclear. According to the literature, PPNT can avoid the use of cytoskeleton inhibitors, and PPN is easy to be found and handled [42]. Thus, it is suggested that PPNT can be used as a novel source of female nuclear material for MRT followed by a brief discussion of potential safety concerns.

Spindle transfer (ST)

The maternal spindle or MII spindle-chromosome complex is formed with oocytes during the second meiotic division. Mitochondria in MII oocyte are spread randomly throughout the cytoplasm, while they are absent in spindles and metaphase chromosome [51]. The method of ST refers to remove the spindle, and transfer it to an enucleated MII oocyte that has had its own spindle removed, followed by fertilization of the reconstituted oocyte [52]. In 2009, ST was first reported in the rhesus monkey by Tachibana *et al.* [51], which shown oocytes had comparable fertilization and blastocyst rates (95% and 61%, respectively) to those of manipulated control oocytes. The same technique has since been performed between mouse oocytes with different mtDNA genotypes, suggesting the rate of blastocyst formation is about 90% and the average mtDNA carryover with reconstituted ST oocytes is less than 1% [53]. According to these animal studies, it is confirmed that ST is technically feasible, with reconstituted oocytes capable of normal fertilization and development to the blastocyst stage at a similar rate as unmanipulated controls. Moreover, ST blastocysts can produce healthy offspring with minimal levels of karyoplasm-derived mtDNA, which confirms the potential of ST to prevent transmission of mtDNA diseases. Therefore, these results in animals encouraged studies in humans where oocyte donors with different mtDNA haplotypes were recruited for mtDNA tracking purpose. Tachibana *et al.* [54] first reported to perform ST in human MII oocytes, which confirmed the feasibility of this technique, however, a significant proportion of human ST zygotes that displayed abnormal fertilization (52%) after Intra-Cytoplasmic Sperm Injection (ICSI). This higher rate of abnormal fertilization in human ST zygotes was thought to reflect premature oocyte activation under suboptimal culture conditions [54]. In the same year, Pall *et al.* [55] performed ST in human MII oocytes, and showed that premature activation of oocytes could be avoided by partial depolymerization of the spindle-chromosome complex through reduced temperature or cryopreservation of oocytes before performing ST. The study also reported that mtDNA carryover is below 1% in reconstituted ST embryos. Recently, the study by Kang *et al.* [56] was the first to perform ST using human MII oocytes from women carrying a pathogenic mtDNA mutation, providing the new evidence to confirm the potential of ST to reduce transmission of an mtDNA mutation in ST blastocysts. This study demonstrates that ST can effectively reduce transmission of a pathogenic mtDNA mutation, and also

suggests that ST blastocysts can give rise to a viable pregnancy and live birth. It is noteworthy, however, ST still has its limitations: 1) ST is highly operator-dependent because spindle in MII oocyte is small and not easy to be found; 2) meiotic spindle-chromosome complexes are vulnerable and prone to be damaged during manipulation; 3) ST also has minor mitochondrial carryout which may result in a high mitochondrial heteroplasmy in some tissues and organs of descendants [57].

Polar body transfer (PBT)

Polar bodies are small haploid cells extruded from oocytes during meiosis [58]. In human, the first Polar Body (PB1) is divided and extruded before ovulation. After fertilization with sperm, zygote would continue to finish the second meiosis and to emit the second Polar Body (PB2). Accumulating evidences recently demonstrate that PB1 and PB2 include the same genetic material and developmental potential as their counterparts inside the ooplasm [57]. PB1 Transfer (PB1T) is defined to remove PB1 from a MII oocyte, followed by transfer to an enucleated MII oocyte with the maternal spindle removed and fertilization of reconstructed oocyte. PB2 Transfer (PB2T) refers to remove PB2 from a fertilized PN-stage zygote and transfer to a recipient zygote with the female pronucleus removed [52]. Both techniques have been successfully performed in animal oocytes, indicating the feasibility of PBT to produce developmentally competent embryos and generate live offspring [59]. PBT has been described recently in the context of MRT [57], because of PBs contain only a few mitochondria that could be easily visualized and handled. Hence, PBT results in lower levels of karyoplast-derived mtDNA when compared to other MRT techniques. What's more, PBT provides a tool to study basic mechanisms of developmental biology related to female meiosis and expands our understanding of genetic stability in oocytes. There is currently only one study of PBT in human MII oocytes, which showed that reconstrured PB1T oocytes were able to normal fertilization by ICSI at a similar rate to unmanipulated controls [60]. PB1T zygotes also had the potential for ongoing development but blastocyst formation was limited, with less frequently (42%) can be developed to blastocysts. The efficiency of Embryonic Stem Cells (ESCs) derived from PB1T blastocyst was low, but further genome-genetic, epigenetic and transcriptional analyses of these ESCs revealed similar DNA methylation and transcriptome profiles when compared to controls [60]. This led the authors to conclude that rescue of PB1T genetic material via introduction into donor cytoplasm may provide a source of oocytes for infertility treatment or MRT for mtDNA disease, although preclinical data is still lacking and the limited embryonic development requires further investigation. Currently, there are no literatures reporting the PB2T in human oocytes, most likely due to the difficulty in distinguishing male and female PNs within human zygotes [57], which makes the procedure technically challenging. As far, it still lacks enough evidences to support that genetic constitute of PBs could be exactly comparable with nucleus in oocyte or zygote [61]. For these reasons, similar to other MRT technologies, PBT will have to meet safety and efficacy requirements of regulatory agencies before approval for routine clinical applications [41].

Ethics challenges for MRT

There is no doubt that MRT may have a positive impact on pregnancy outcome of ART in older women. However, current MRT technologies are associated with noteworthy theological, ethical, as well as safety concerns. It is still hard for us to estimate the consequences caused by mitochondrial heterogeneity, though MRT has minor mitochondrial DNA carryout and maternal inheritance. In the case of PNT, abundant mtDNA would augment and intensify after fertilization, thereby contaminating fresh mitochondria and then resulting in a high heterogeneity level, while ST is highly operator-dependent and not easy to generalize and it also have minor mitochondrial carryover [57]. There is no certainly evidence that PBT may not cause mitochondria carryout. Traces of deficient mitochondria and mtDNA would be markedly amplified in the progress of oocyte maturation and spread among the whole body during embryonic development. Accordingly, these incomplete replacement therapies may also generate abundant mitochondria remnant that results in a significant loss of lifespan and many serious inheritable disabilities [62-64]. In theory, PNT or PB2T has performed with an individual fertilized embryo. It is therefore also necessary to consider the ethical implications these techniques might have if PNT or PB2T eventually used to replace mitochondria. Current law defines this 'germline modification' as illegal and ethic concepts disapprove the behavior of modifying other's genes. In January 2017, the UK became the first country to license the clinical use of an MRT when the Newcastle Fertility Centre was granted a license by the Human Fertilization and Embryology Authority to use PNT. However, this was only after the first baby born through MRT had been revealed to the world in 2016. Subsequently, the license

is only permitted to cure those who are identified that offspring will have serious mitochondrial genetic diseases in the UK [39]. We argue that MRT can be used to prevent mitochondrial diseases, but do so with the view that they should be used cautiously in ART. It is also likely that MRT will be accompanied in the clinic by some broader advances in biomedicine, including the emergence of CRISPR/Cas9-mediated genome editing (such as'He Jiankui' accidence [65]). In light of the remaining ethical challenges that still exist surrounding MRT, we maintain that the future use of MRT still requires a concerted effort among the global research and clinical community to put in place responsible innovation and governance of these techniques. In this context, it follows that the search for alternative approaches for improving the oocyte quality and prevention of mtDNA disease must continue. If capturing the promise of novel embryo-sparing, donor-independent technologies, it will require thorough preclinical validation of safety and efficacy prior to any consideration of first-in-human clinical trials [66].

A successor of MRT: mitochondrial transfer therapy

In order to avoid the ethic disputed on '3 parents' babies', it has been paid more and more attention in transferring autogenous mitochondria to oocyte to improve oocyte quality [67]. This method origins from the Ooplasmic Transfer (OT), which transfer ooplasm from donor eggs at Metaphase II (MII) stage into patient MII eggs [68]. After the first successful clinic treatment of OT, it has verified that the "mitochondrial supplement therapy" is effective [69]. But ooplasm contains numerous impurities expect mitochondria, and the mitochondria used for transferring are heterogenous. To evade the unknown risk carrying by third-party genetic materials, transferring autologous mitochondria is necessary. The method of autogenous mitochondria transfer involves isolation of mitochondria

from autologous cell germlines such as granular and cumulus cells, oogonial stem cells or mesenchymal stem cells from diverse tissues and injects to oocyte by ICSI (Figure 3). Recent researches reveal that this method does works. It has been shown that transferring autologous mitochondria to oocyte could promote oocyte quality and improve the fertility in aged mice [70]. In early 2015, dozens of couples had successful outcomes benefiting from the treatment of autologous mitochondrial transfer, i.e. Autologous Mitochondrial Energy Transfer (AUGMENT) [71]. Although initial descriptive studies seemed promising, a recent prospective study demonstrated that AUGMENT technique does not seem to improve embryo quality in infertile patients with premature ovarian ageing and a background of poor embryo quality in previous IVF cycles [72]. Moreover, there were no significantly difference in the ratio of euploid embryos obtained per injected MII and per fertilized oocyte between groups, suggesting that the injection of the extra volume of mitochondria suspension during ICSI did not damage oocyte membrane integrity [72]. The AUGMENT treatment was not able to modify the dynamics of mtDNA content of the human blastocysts, with no differences observed when compared to the control group, at least at the trophectoderm cell level. According to the above results, it is important to point out that there is no necessary for us to perform the nuclear genome by mitochondrial transfer therapy technology, which may be helpful to dramatically reduce risk of chromosome damage and the difficulty of operation and the operating difficulty. On the one hand, transferring functional mitochondria to sluggish oocyte could provide enough energy for them to develop as normal. On the other hand, diverse metabolism-related and cell survival pathway would be activated, then initiating the quiescence mitochondria and prevent oocyte from apoptosis. Compared with replacement therapy, the advantage of mitochondrial transfer therapy is able to overcome the inherited bottleneck. However, this method now remains controversial and immature. Firstly, autologous stem cells are valuable and uneasy to acquire. Secondly, methods for isolating vital mitochondria from cell cultures are immature. It's difficult for us to analyze the number and complete function of isolated mitochondria, while the dosage and quality of transferring mitochondria will also make a great contribute to the success. Thirdly, this technology lacks firm theoretical foundation. The detailed internal mechanism of this therapy is now yet not clear. There is still little known about exactly how the complementary mitochondria work in low-quality oocyte and how mitochondria cooperate with nucleus to regulate cellular proliferation and to refresh oocyte. Thus, it is recommended that this transfer therapy should not currently used for infertility treatment, and further investigations are needed in order to better understand the efficacy and safety of this technology.

Conclusion and Perspectives

The role of mitochondria in oocytes is becoming increasingly clear, which in case of mitochondrial dysfunction may cause decreased fertility and increased risk of aneuploidies. Importantly, oocyte mitochondria accumulate errors and become dysfunctional with aging, most likely resulting in infertility in women of advanced age. Therefore, mitochondria have been proposed as a biomarker of fertility to be assessed during IVF according to detection the number of mtDNA in oocytes and embryos. Also, various strategies have been performed to increase the mitochondria content in order to improve the quality of oocytes in older women, which have provided promising evidence of fertility rescue. Once its effectiveness and safety are appropriately confirmed, mitochondrial replacement/transplantation therapies will have great potential to be used as a novel treatment in clinics of assisted reproduction. Altogether, alternate and innovative approaches are being proposed but remain in very early stages. Hence, the search for an effective solution to improve oocyte quality continues.

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