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Perspective

Mitochondrial replacement techniques or therapies (MRTs) to improve embryo development and to prevent mitochondrial disease transmission

Xiang-Hong Ou [a], Qing-Yuan Sun [a, b, c, *]

[a] Reproductive Medicine Center, The 2nd People's Hospital of Guangdong Province, Guangzhou 510317, China
[b] State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[c] University of Chinese Academy of Sciences, Beijing 100101, China

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The mitochondrion which contains its own double-stranded circular DNA is a semi-independent organelle that plays critical roles in cell activity. Mitochondrial DNA (mtDNA) is maternally inherited through several mechanisms that have been proposed (Luo et al., 2013) and, if mitochondrial mutations are inherited to the offspring, it is possible to cause mitochondrial diseases such as neuropathy, cardiomyopathy, myopathy, and liver failure. In patients with mtDNA diseases, either all copies of mtDNA are mutated (homoplasmy), or a large proportion of mutated mtDNA co-exists with wild-type mtDNA (heteroplasmy) (DiMauro and Schon, 2008). Clinically, most maternally inherited mitochondrial diseases are caused by heteroplasmy, and only a high proportion (usually greater than 60%) of mutated mtDNA in affected tissues will cause diseases (Taylor and Turnbull, 2005). Until recently, limited success has been achieved in developing effective treatment for mtDNA diseases.

It is well known that age-associated decline in female fertility is largely due to poor oocyte quality. Both nuclear anomalies and cytoplasmic mitochondrial dysfunctions contribute to decreased oocyte quality. Mitochondria play a central role in determining oocyte quality and their functions are adversely affected by ageing. Although the oocyte has surveillance mechanisms for eliminating mutated mtDNA to some extent, and thus preserving mitochondrial integrity over the generations, ageing-related mitochondrial mtDNA mutations do accumulate in the oocyte, which deteriorates the oocyte developmental competence and increases the risk of abnormal mitochondria transmission to the offspring (May-Panloup et al., 2016).

Various reproductive technologies have recently been designed to partially or fully replace oocyte mutated mtDNA to improve embryo development and reduce the risks of mother-to-child mtDNA disease transmission. These recent strategies raise hopes for prevention of mtDNA disease transmission, and for improving development of embryos from aged women. Here, we will discuss the related progresses, advantages, disadvantages, and debates on these mitochondrial replacement techniques or therapies (MRTs).

1. Ooplasm or mitochondrial transfer

The feasibility of transferring mitochondria between oocytes in attempts to improve ATP production and developmental potential of the recipients was first reported 20 years ago. Either partial ooplasm or isolated mitochondria can be injected into recipient oocytes for improving oocyte quality or for mtDNA mutation therapy. Ooplasm transfer was first introduced as potential approach for treating women who suffered from repeated embryonic development failure due to defective ooplasm with insufficient mitochondrial energy supply (Cohen et al., 1997, 1998; Van Blerkom et al., 1998). This manipulation has been proved to improve embryonic quality, reduce fragmentation, increase the cleavage rate, and allow successful implantation and development. Ooplasm transfer had led to the birth of nearly 30 babies by 2001. However, ooplasm transfer to prevent mitochondrial diseases was in doubt, since only a small amount (10%–15%) of donor ooplasm was transferred to the recipient's oocyte. This manipulation was then suspended by Food and Drug Administration (DFA), because mixing of human ooplasm from two different women may generate mitochondrial heteroplasmy (Barritt et al., 2001). Caution has been voiced about using such a technique, due to the absence of basic preclinical research and suitable experimental controls and thus unproved efficacy and safety (Bredenoord et al., 2008). On the other hand, ooplasm transfer also raises ethical issues, since the tri-parental embryos consist of genetic materials from both the

* Corresponding author.
E-mail address: sunqy@ioz.ac.cn (Q.-Y. Sun).

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couple's germ cells and the donated oocyte.

2. Germinal vesicle transfer

Cytoplasmic transfer or mitochondrial transfer can only partly substitute the functional mitochondrial population. To largely replace the mitochondria of an oocyte, a reliable technique of nuclear transfer for immature mouse oocytes was established (Takeuchi et al., 1999; Liu et al., 1999). When the germinal vesicle (GV) of one oocyte is transferred into the ooplasm of another enucleated oocyte, the reconstructed oocyte can complete *in vitro* maturation, fertilization, early embryo development, and finally lead to live birth after embryo transfer in the rabbit and mouse (Li et al., 2001; Liu et al., 2003). The GV transfer has been proved to improve the quality of human oocytes from women with advanced age (Zhang, 2015). In one study, 4 out of 7 human oocytes reconstructed by transferring GVs of aged oocytes into cytoplasts of young oocytes extruded a normal first polar body (PB1) (Zhang et al., 1999). However, it was later reported that chromosome misalignment could not be rescued by aged mouse GV transfer into the ooplasm of young mice (Cui et al., 2005).

Since the mitochondria surrounding the GV are easily carried over into the reconstructed oocytes, it is impossible to avoid mitochondrial heteroplasmy, which is another challenge for its application. The third challenge for GV transfer technology is that cumulus cells surrounding the oocyte need to be removed before microinjection, which may negatively influence oocyte developmental potential.

3. Spindle apparatus transfer

MII spindle transfer was reported nearly 20 years ago in mice (Wang et al., 2001). The MII oocyte meiotic apparatus or spindle was transferred between different strains of mice, and viable offspring were produced. Successful spindle-chromosomal complex transfer in mature non-human primate (*Macaca mulatta*) oocytes was then reported (Tachibana et al., 2009).

In the human, the first live birth was recently produced by using oocytes reconstructed by spindle transfer for mtDNA mutation rescue. The spindle apparatuses from oocytes of a woman suffering from Leigh syndrome with mtDNA mutation 8993T > G, which caused multiple undiagnosed pregnancy losses and offspring deaths, were transferred into enucleated donor oocytes, and a male euploid blastocyst was obtained from the reconstructed oocytes, having only a 5.7% mtDNA mutation load. Transfer of the embryo led to the birth of a boy with 2.36%–9.23% mtDNA mutations in the tested tissues (Zhang et al., 2017a). Kang et al. (2016) recently also reported spindle transfer outcomes in several families with common mtDNA syndromes such as Leigh syndrome. Spindle transfer produced embryos containing >99% donor mtDNA. However, some embryonic stem (ES) cell lines from these embryos showed gradual elimination of donor mtDNA and replication priority of original maternal mitochondria.

By studying conplastic mouse strains which are developed by backcrossing the nuclear genome from one inbreed strain into the cytoplasm of another, it was shown that mtDNA match with the nuclear-encoded mitochondrial genes (mtDNA haplotype) is important for various physiological factors such as reactive oxygen species generation, insulin signaling, obesity, and ageing parameters, and thus health longevity (Latorre-Pellicer et al., 2016). It appears that compatibility between donor mtDNA and nuclear DNA is necessary, and thus selecting compatible donor mtDNA may be required when mtNDA replacement technology is applied.

4. Pronuclear transfer

An alternative approach to replace oocyte mitochondria is to transplant a pronuclear karyoplast into the perivitelline space of an enucleated fertilized egg, and then fuse the karyoplast with the recipient ooplasm with donated mitochondria to obtain a reconstructed zygote. This approach was first reported in mouse zygotes long time ago (McGrath and Solter, 1983) and it was shown that mtDNA-related phenotypes were corrected (Sato et al., 2005), but this manipulation was only conducted recently in the human. Pronuclear transfer could be used to treat patients who have arrested embryo development after *in vitro* fertilization (IVF). Recently, Zhang et al. (2016) conducted zygote nuclear transfer in a woman whose embryos were arrested at the two-cell stage, and successfully obtained a viable pregnancy. Fetal mtDNAs were identical to those of donated mitochondria, with no detection of the patient's mtDNA (Zhang et al., 2016). Craven et al. (2010) used abnormally fertilized human zygotes to study the feasibility of preventing transmission of mtDNA from mother to child by pronuclear transfer, and found that pronuclear transfer only carried over less than 2% of mtDNA with two pronuclei, which is far below the threshold that causes mitochondrial diseases. This is also far below the mtDNA carry-over proportion in pronuclear transfer mice which had 5%–33% heteroplasmy at day 300 after birth (Sato et al., 2005). It was recently shown that transfer of a pronuclear karyoplast shortly after fertilization was more effective in terms of blastocyst development than late-stage pronuclear transfer, and reduced mtDNA carry-over proportion to less than 2% in 79% of the blastocysts (Hyslop et al., 2016).

When stem cell lines derived from the pronuclear transfer blastocysts were analyzed, a progressive increase in heteroplasmy in some stem cell lines was observed (Hyslop et al., 2016). One of possible reasons is that mtDNA with the karyoplast may have a replication advantage over mtDNA in recipient ooplasm. It is very possible that pronuclear transfer human offspring may show higher mtDNA heteroplasmy than early embryos. Therefore, pronuclear transfer may be a potential manipulation to improve embryo development or to reduce the risks of mtDNA diseases, but this may not guarantee complete prevention of mutated mtDNA transmission as GV or spindle transfer does.

5. Polar body transfer

During the first and second meioses of oocytes, homologous chromosomes and sister chromatids are equally segregated, respectively, into the small first polar body and second polar body, and to their counterpart, the oocyte. Nearly 20 years ago, it was shown that normal offspring could be produced by polar body transfer into enucleated mouse oocytes (Wakayama and Yanagimachi, 1998). Due to the small size, the polar body contains only a very small amount of cytoplasm and mitochondria, and polar body transfer would carry over minimal mtDNA to the enucleated oocyte (Wei et al., 2015). Wang et al. (2014) performed polar body transfer, aiming to minimize the mtDNA carryover in mice. They showed that the F_1 generation from polar body transfer contains minimal donor DNA carryover compared to spindle transfer and pronuclear transfer (Wang et al., 2014). Functional human oocytes were also generated by polar body transfer (Ma et al., 2017). Recently, polar body transfer was tested to reconstruct human embryos for potential MRT (Wu et al., 2017). Polar body transfer also supported the oocyte development to blastocyst stage in a case of repeated embryo fragmentation (Zhang et al., 2017b). These data suggest that polar body transfer could be a potential MRT for mtDNA disease prevention.

6. Autologous mitochondrial transfer

Accumulation of mtDNA mutations in oocytes may lead to the decline of fertility with age, and it may have deleterious consequences for the offspring, which could be reversed by the introduction of wild-type mtDNA into females. The GV transfer, spindle transfer, polar body transfer and pronuclear transfer may replace most but not all dysfunctional mitochondria, while ooplasm transfer only replaces a small proportion of mitochondria. All these heterologous mitochondria transfer procedures cannot completely avoid the so-called tri-parental ethnic issues.

Autologous mitochondrial transfer cannot be used to treat mitochondrial diseases, but it may have a value for improving oocyte quality by increasing intra-egg mitochondria number and thus ATP production. Mitochondrial transfer from self-granular cells was reported to improve human embryo quality in aged women, and led to production of offspring in women with repeated abortions (Kong et al., 2004). However, it is hard to evaluate the efficacy due to the absence of a control, and more importantly, injected mitochondria from autologous granular cells may also contain mtDNA mutations.

Although controversial, adult oogonial stem cells were reported to exist in mice and humans, which opens a new path for transfer of healthy mitochondria from these cells without the need for young donor eggs (Woods and Tilly, 2015). Recently, oogonial precursor cell-derived autologous mitochondria injection was found to improve oocyte quality and IVF outcomes in women with multiple IVF failures (Oktay et al., 2015). However, the limited source of oogonial precursor cells may limit its application. Mitochondria from other types of autologous stem cells or iPS (induced pluripotent stem) cells may also be considered in the future. Another possible choice is to obtain healthy autologous mitochondria from oocytes obtained by *in vitro* follicle activation and culture of patient's ovarian cortical biopsies or from surplus immature oocytes collected from women undergoing assisted reproductive technology (ART), which would potentially improve oocyte quality (Kristensen et al., 2017).

7. Conclusion and perspectives

The mitochondria replacement by transfer of heterologous ooplasm, GV, spindle, polar body, and pronuclei has been tested in animals and humans to improve developmental potential of aged defective oocytes or to prevent trans-generational mitochondrial disease transmission, but clinical translation of these techniques requires further validation for their efficacy and safety. In addition to ooplasmic transfer which substitutes only a small portion of mitochondria, all the other MRTs replace most but not all dysfunctional mitochondria, thus cannot completely solve the problems of mtDNA heteroplasmy and tri-parental ethical issues. The small amount of carry-over defective mitochondria with the karyoplast during MRT manipulation may not cause mitochondrial diseases of offspring, but we need to remember that mtDNA with the karyoplast has been found to have a replication advantage over mtDNA in recipient ooplasm, which may have a long-term effect. Human zygotes and cleaving embryos derived from spindle transfer contain more than 99% donor mtDNA, but ES cell lines derived from MRT blastocysts when different donors are employed do show different levels of mtDNA heteroplasmy, and a few cell lines contain high levels of maternal mtDNA, and extended passaging causes a complete loss of donor mtDNA (Kang et al., 2016). Thus, compatibility between donor mtDNA and recipients should be considered when MRT is applied. Recently, pregnancy was achieved by pronuclear transfer to overcome embryo development arrest and babies were obtained by spindle nuclear transfer to prevent mitochondrial disease transmission (Zhang et al., 2016, 2017a), but the application of MRTs in clinical human assisted reproduction has been in debate. Autologous MRT may solve the ethical issue and mtDNA hetroplasmy problems, but it is hard to be used to prevent trans-generational transmission of mitochondrial diseases. MRT using mitochondria from autologous oogonial precursor cells has been conducted, but the existence of oogonial stem cells, the limited cell source and technical difficulty for its expanded clinical application is being debated. Autologous mesenchymal stem cells or iPS cells, and even *in vitro*-grown oocytes, may provide non-mutated mitochondria for transfer, which may be considered in the future.

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