Mitochondrial replacement therapy and the "three parent baby"

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Abstract

The mitochondria contained in eukaryotic cells have their own DNA, and heritable mutations in mitochondrial DNA (mtDNA) can cause a variety of disorders in humans. A new therapy, mitochondrial replacement therapy (MRT), is currently being developed to address these mitochondrial disorders by eliminating the mutated mtDNA from the germline. The two main MRT techniques are pronuclear transfer, conducted in the zygote after fertilization, and spindle-chromosomal complex transfer, conducted in the oocyte before fertilization. In pronuclear transfer, the pronuclei from a zygote affected by a mtDNA mutation are transferred to an enucleated normal zygote. In spindle-chromosomal complex transfer, the genetic material from an oocyte affected by a mtDNA mutation is inserted into the cytoplasm of a donor oocyte that contains healthy mitochondria. A third method, polar body genome transfer, attempts to increase the efficiency of the above techniques by using polar bodies to supply the genetic material. While MRT is legally and ethically controversial, it has recently been implemented successfully in a clinical setting.

Keywords: Mitochondrial replacement therapy; Mitochondrial disease; mtDNA; Gene therapy; Assisted reproductive technology; Three parent baby.

1. Mitochondria

Mitochondria are the organelles in the cell that are primarily responsible for generating energy for the organism through the electron transport chain. They are ubiquitous in the body tissues and gametes, and can undergo fusion and fission (Karp 2010).

1.1 Endosymbiotic theory

One of the most widely accepted evolutionary explanations of how prokaryotic mitochondria came to be a part of the larger human eukaryotic cell is endosymbiotic theory, initially proposed by Lynn Margulis in the 1970s. In its most basic terms, the theory suggests that an ancestor of the modern mitochondrion was taken into a larger prokaryotic cell body, but was not consumed, as it provided benefits to the larger cell. The prokaryote offered optimized production of energy to the larger cell (generating more adenosine triphosphate [ATP] per mole of glucose used), and the larger cell provided protection for the prokaryote. This mutually beneficial arrangement has survived millions of years of evolution (Margulis 1981). Due to this developed partnership, each cell contains two types of DNA: nuclear and mitochondrial.

1.2 The mitochondrial genome

Circular and double-stranded, the mitochondrial genome is 16,569 bp (base pairs) in length (Taanman 1999), minute in comparison to the much larger 3.2×10^9 bp nuclear genome (Makalowski 2001). Although the mitochondrial genome bears many similarities to its ancestral prokaryotic genome, it is not self-replicating (likely due to the mitochondrion's increased dependence on its larger host throughout evolution). Rather, the mitochondrial genome relies on nuclear DNA to provide the machinery for replication and transcription, but provides its own machinery for translation. Mitochondrial DNA (mtDNA) contains 37 genes. These genes code for two ribosomal RNAs (rRNAs), 13 polypeptides, and 22 transfer RNAS (tRNAs) (Taanman 1999). The tRNAs aid in decoding the mRNA transcript into amino acids, and the rRNAs form the ribosomes that link amino acids to create polypeptides. Although the contribution of mtDNA is very small in comparison to that of nuclear DNA, its dysfunction can have major consequences.

1.3 Mitochondrial DNA mutation rate

The exact rate of mtDNA mutation in humans is not definitively known. In *Drosophila melanogaster* (the common fruit fly), the mutation rate of mtDNA is on average 10 times higher than the mutation rate of nuclear DNA. Haag-Liautard et al. (2008) estimated the mtDNA mutation rate (per site per generation) in *D. melanogaster* at 6.2×10^{-8}. *D. melanogaster* is often used as a model for genetic investigations in other organisms, including humans.

Accordingly, although exact mutation rates for human genes have not been found, the rate in *D. melanogaster* has been provisionally applied to humans. Moreover, mtDNA is particularly susceptible to G ↔ A mutations on the sense strand. It has a mutation rate 70 times higher than the nuclear strand in these hotspot regions (Haag-Liautard et al. 2008). The reasons driving variation in mutation rates are unclear. Two hypotheses are the generation time hypothesis and the metabolic rate hypothesis. The generation time hypothesis proposes that the shorter generation time of mitochondria leads to more replication errors over a set amount of time (compared to other types of DNA), simply because more replications occur. The metabolic rate hypothesis proposes that mitochondrial mutations occur at a higher rate as a result of the reactive oxygen species released during normal metabolic respiration (Nabholz et al. 2008.) Fewer DNA repair systems appear to be present in mitochondria, contributing to the high mutation rate (Karp 2010).

1.4 Mitochondrial inheritance

Due to its separate evolutionary pathway, mtDNA does not have the same inheritance pattern as nuclear DNA. While nuclear DNA has both maternal and paternal contributions, mtDNA exhibits a matrilineal inheritance pattern, meaning that it is passed only through the mother (Karp 2010). Males can inherit these mutated mitochondria and resultantly suffer from mitochondrial diseases, but they cannot pass mutations on. Females can both inherit these mutations and pass them on to their offspring. Mitochondria self-replicate from pre-existing mitochondria, and therefore, any mutations that arise may also replicated.

1.4.1 Heteroplasmy

This inheritance pattern leads to mitochondrial heteroplasmy (or the presence of multiple kinds of mitochondrial DNA in a single cell), which can result in a mixture of healthy and unhealthy mitochondria in each cell. Their effect on the organism depends on the proportion of healthy to unhealthy mitochondria. Oocytes are the maternal contribution to the offspring, and when oocytes are created, the mitochondria are not divided evenly. If there is a mutation in the mtDNA, the mitochondria present in the newly formed ovum may exist in a different proportion of healthy to unhealthy than in the mother. This is referred to as the bottleneck effect. Mitochondrial mutations inherited in this manner result in three phenotypes: normal (carrying none of the mutated mitochondria), carrier (having mitochondrial heteroplasmy but in a small enough proportion to result in an overall healthy phenotype), and affected (either having heteroplasmy with a large enough proportion of unhealthy mitochondria to display symptoms of an unhealthy phenotype, or homoplasmy). The proportion of mutated mtDNA often correlates with the severity of mitochondrial diseases (Karp 2010).

2. Conditions associated with mitochondrial DNA mutations

A variety of mitochondrial diseases can occur as a result of mtDNA mutations. These diseases tend to impact muscle and brain function due to the high ATP usage of these processes (Karp 2010). The following examples of mitochondrial diseases provide an overview of some of the mutations that can cause a disease phenotype, and the variable severity of the resulting diseases.

2.1 Mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like episodes

Mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS) is one such condition that can be caused by a mutation in one of multiple mitochondrial genes. The most common mutation causing MELAS is found in tRNA gene MT-TL1. This tRNA gene is responsible for adding a leucine to the growing protein. A malfunction in this tRNA can result in a malformed protein or a sub-optimal rate of protein production, causing symptoms associated with MELAS. MELAS has an immense impact on those who suffer from it, and symptoms include muscle pain and weakness, seizures, and stroke-like episodes. These stroke-like episodes mimic true strokes in their associated symptoms, and over time can damage the brain permanently. Permanent brain damage may result in decreased intellectual function, vision problems, and mobility difficulties (National Library of Medicine 2016a).

2.2 Leber hereditary optic neuropathy

Leber hereditary optic neuropathy (LHON) occurs as a result of mutations in mitochondrial genes such as MT-ND1, MT-ND4, MT-ND4L, and MT-ND6. Mutations in these genes, however, do not necessarily lead to symptoms of LHON. The majority of those affected by LHON never experience symptoms. It is possible, however, that LHON is caused by the interplay between these identified genes and other genes, or that environmental factors contribute to the appearance of symptoms. The primary symptom of LHON is vision loss due to optic nerve cell death. Although the global incidence of this disease is unknown, approximately one in 30,000–50,000 in Finland and northeast England are affected by it (US National Library of Medicine 2016b). However, as these mutations are often asymptomatic, it is difficult to ascertain exactly how many people have the mutations in their mtDNA.

2.3 Leigh syndrome

Leigh syndrome is most commonly caused by a mutation in the gene MT-ATP6, which encodes a component of the oxidative phosphorylation pathway that produces energy. This mutation leads to cell death. Lesions on the brain are commonly found in those affected, and common symptoms include a progressive loss of mental abilities and

of mobility. Approximately one in 40,000 infants are affected by Leigh syndrome, with some regions, such as Saguenay Lac-Saint-Jean in Quebec, Canada, having a much higher incidence of one in 2,000. In children affected by Leigh syndrome, death usually occurs within three years (National Library of Medicine 2016c).

3. Mitochondrial replacement therapy

Approximately one in 4,000 people worldwide suffer from a mitochondrial disease (National Library of Medicine 2016a). There is, moreover, evidence to suggest that one in 200 people have a pathogenic mtDNA mutation (Elliott et al. 2008). As conditions such as those detailed above result from mtDNA mutations, researchers have directed their efforts toward a therapy that takes advantage of the mitochondrion's independent nature. The goal of this therapy is to produce healthy offspring that remain genetically related to the affected mother.

3.1 Techniques involved in mitochondrial replacement therapy

There are 2 primary methods used to perform MRT, pronuclear transfer and spindle-chromosomal complex transfer, respectively. Additionally, a promising third method, polar body genome transfer, is currently in development. The premise of these techniques is to transfer the genetic material from the affected oocyte or zygote to an oocyte or zygote that contains healthy mitochondria. In theory, this would remove the mutated mtDNA and allow the mother to give birth to a healthy child without the risk of passing on the mutation. Due to the relatively independent nature of the mitochondrion, this replacement would ideally eliminate the mitochondrial mutation from the germline while minimizing the third party's genetic contribution.

3.1.1 Pronuclear transfer

Pronuclear transfer involves transferring the pronuclei contained in a zygote affected by mutated mtDNA to an enucleated normal zygote. The zygote forms after the sperm has entered and fertilized the oocyte. This zygote is then implanted, and a normal pregnancy is expected. A successful rescue experiment using pronuclear transfer was performed in mice by Sato et al. (2005). Using "transmitochondria mice" (or mito-mice) affected by respiratory defects due to deletions in the mtDNA (such as those observed in MELAS), their method was as such:

1. Oxygen consumption rates were measured in the affected mice before manipulation. Mutated mtDNA proportions from a variety of tissues in neonates and adult mice were measured by Southern blot to detect specific DNA sequences. Real-time monitoring polymerase chain reaction (PCR) was used to track DNA amplification. Blood lactate concentration and blood urea nitrogen were also measured.
2. Oocytes and second polar bodies (the small cells which bud off from an oocyte, but do not become ova) were collected from both diseased and healthy mice. Second polar bodies were used to determine heteroplasmy in the embryos.
3. Karyoplasts (the nuclei surrounded by small amounts of cytoplasm) from mito-mice zygotes were transferred into enucleated normal zygotes.
4. The pronuclei of the mito-mice and the normal zygotes were fused by electric pulse and transferred to pseudopregnant female mice (those which are not pregnant, but are bodily prepared to bear offspring, typically due to copulation with a sterile mate)
5. Diagnosis of mtDNA genotypes was determined by examining second polar bodies and tail biopsy samples, both after fertilization and postnatally.
6. Blood lactate concentration and blood urea nitrogen were measured in the F_0 generation (that is, the first generation of offspring born from the mice on which the experiment was conducted), and they were observed until death.

Non-transplanted mito-mice died at 218-277 days, whereas all mito-mice that had undergone pronuclear transfer lived for more than 300 days, at which point they were terminated. These results indicate that the mice that underwent pronuclear transfer did not die from respiratory mitochondrial diseases, and that the rescue experiment was successful (Sato et al. 2005).

After the success of the previous experiment, a similar pronuclear transfer experiment was performed in humans. Craven et al. (2010) used unipronuclear and tripronuclear zygotes (abnormally fertilized and not fit for the in vitro fertilization [IVF] process) from an in vitro fertilization clinic for this experiment, with informed consent from the donors. The methods they employed were as follows:

1. Pronuclei were removed from donor zygotes.
2. The donor pronuclei were placed in enucleated recipient zygotes. They were fused using inactivated viral envelope proteins, and cultured for 6-8 days.
3. Microsatellite markers (or tracts of repetitive DNA that act as DNA fingerprints) confirmed that the fused zygotes contained the nuclear genetic material of the donor zygotes.
4. The zygotes were observed for development in vitro to determine whether the manipulation was compatible with life.
5. Sequencing and restriction fragment length polymorphisms (RFLPs), which allow for the detection of single nucleotide polymorphisms in the DNA by changing restriction enzyme cut sites (or the specific recognition sequences at which restriction enzymes cut the DNA), were used to determine the carryover rate of mtDNA from the donor.

Craven et al. initially allowed some cytoplasm from the donor to be transferred into the recipient zygote, which yielded 8.1% mtDNA carryover. A second experiment was performed, carrying over a minimal amount of cytoplasm from the donor. This yielded a much lower percentage, 1.7%, of mtDNA carryover (Craven et al. 2010). This difference is important, as any mutated mtDNA carried over from the donor zygote can potentially cause an affected phenotype in the offspring, defeating the purpose of the manipulation. As this experiment used abnormally fertilized zygotes, it is difficult to ascertain the exact survival rate in normally fertilized zygotes. The experiment conducted by Craven et al. yielded a 50% zygote survival rate after the manipulation had been performed, but it is unclear how many of the zygotes would have survived without the manipulation.

3.1.2 Spindle-chromosomal complex transfer

Spindle-chromosomal complex transfer involves isolating the nuclear genetic material, in the form of the spindle-chromosomal complex, from an affected oocyte, and transferring it into a healthy enucleated oocyte. The spindle-chromosomal complex is isolated when the cell is in metaphase. In metaphase, the chromosomes are aligned in the center of the cell, with the spindles at each end. The healthy oocyte is then fertilized and implanted, similar to a typical IVF procedure (Mitalipov and Wolf 2014).

Tachibana et al. (2009) demonstrated that this procedure could successfully be carried out in the oocytes of rhesus macaque monkeys (*Macaca mulatta*). Their methods and results were as such:

1. Using MitoTracker Red labelling and subsequent confocal laser scanning microscopy (which allows for the visualization of cells), they confirmed that the spindles and metaphase chromosomes contained no mitochondria.
2. The karyoplast, which contained the nuclear genetic material from the donor oocyte, was fused with the enucleated oocyte using electroporation (whereby a pulse of electricity is used to open pores in cells, allowing DNA through).
3. The developmental potential of the fused oocytes was determined. After intracytoplasmic sperm injection (ICSI), blastocysts were analyzed by labelling with 4', 6-diamidino-2-phenylindole (or DAPI, a compound that stains DNA) and NANOG (an antibody that recognizes the Nanog protein in inner cell mass [ICM]). The total cell count and ICM cell count of the experimental group of blastocysts were comparable to the control group, indicating normal developmental potential. To further characterize developmental potential, Tachibana et al. isolated embryonic stem cell lines from the manipulated blastocysts. These cell lines were able to differentiate normally. Giemsa staining (used to visualize different regions of the chromosome) of these cell lines was conducted, revealing normal karyotypes with no abnormalities.
4. The blastocysts were implanted into female rhesus macaque monkeys and normal gestation occurred in the three monkeys that became pregnant. The three monkeys born at the time of the study's publication were healthy, and had normal birth statistics.
5. The nuclear DNA and mtDNA were examined in the three monkeys born through this procedure. Using microsatellite markers, it was determined that all offspring inherited their nuclear DNA exclusively from the spindle donor. Three techniques were employed to analyze the mtDNA content: direct sequencing, RFLPs, and real-time PCR. The RFLP and real-time PCR tests, which can detect heteroplasmy at the level of three, did not detect any heteroplasmy in these offspring.

One problem that arose during these manipulations occurred during the fusion of the spindle-chromosomal complex. Electroporation caused the oocytes to resume meiotic division and either to progress to anaphase II or to complete meiosis prematurely. This is incompatible with the IVF process. In normal oocytes, meiosis II is arrested in metaphase and proceeds upon fertilization by the sperm due to a calcium ion flux, triggering calmodulin to become active (Gilbert 2000). A premature progression to anaphase II inhibits the fertilization process and thus would not result in the production of offspring. As an alternative, Tachibana et al. (2009) used an extract from Sendai virus (SeV) to fuse the spindle-chromosomal complex with the enucleated oocyte, and this method successfully allowed for fusion without the progression to meiosis II.

The monkeys born as a result of the 2009 experiment were re-examined in 2013. The purpose of this re-examination was to determine whether a mismatch in nuclear and mtDNA could cause mitochondrial dysfunction in the offspring born through spindle-chromosomal complex transfer. By all measures, most notably ATP levels and mitochondrial membrane potential, offspring were normal and matched the controls. Additionally, in the blood and skin samples taken, there were no significant changes in mtDNA carryover and heteroplasmy (which could cause the disease phenotype later in life).

Following the success of the initial experiment on rhesus macaque monkeys, Tachibana et al. (2013) conducted spindle-chromosomal complex transfer in human oocytes. Their methods were as follows:

1. Mature metaphase II oocytes were taken from volunteers who underwent ovarian stimulation. For the experiment, 65 oocytes were manipulated, and 33 were used as non-manipulated controls.
2. In the manipulated oocytes, spindle-chromosomal complex transfer was completed. Of the manipulated oocytes, 94% survived fertilization by intracytoplasmic sperm injection, and 73% formed

pronuclei. These numbers were comparable to the controls (97% and 75%, respectively). Abnormal fertilization was much higher in the manipulated group than in the control group (with a 13% abnormal fertilization rate in the experimental group). Blastocyst formation rate was comparable to the controls in the normally fertilized experimental group.

3. Embryonic stem cell (ESC) lines were derived from the blastocysts. The experimental ESC lines were identical to the controls in morphology. They demonstrated pluripotency (the ability to form many different types of tissue) when injected into immunodeficient mice, forming teratoma tumors (tumors composed of multiple different types of tissue).
4. The rate of mtDNA carryover was examined in both experimental embryos and cell lines. Using a RFLP assay and ARMS-qPCR (amplification-refractory mutation system quantitative PCR, which uses real-time amplification of DNA to identify mutated sequences), it was determined that there was only negligible carryover of mtDNA.
5. Giemsa staining was performed, determining that the normal experimental embryos contained normal diploid male or female karyotypes. No numerical or structural abnormalities were detected.

The abnormal fertilization of some of the experimental oocytes was attributed to the premature progression of meiosis II, a result of the spindle-chromosomal complex transfer manipulations in the oocytes. Some experimental oocytes had progressed from the arrested metaphase II to anaphase II (as in the experiment by Tachibana et al. in 2009, such progression interfered with proper fertilization), whereas all control oocytes remained in metaphase II until fertilization.

Tachibana et al. (2013) also explored the effects of oocyte cryopreservation (preservation by freezing) on spindle-chromosomal complex transfer and blastocyst development. (This is a pertinent topic, as some who experience fertility difficulties choose to preserve their oocytes in this manner. Oocyte cryopreservation would also make the process of spindle-chromosomal complex transfer much more viable for those undergoing IVF treatment. Same-day retrieval of oocytes, however, is difficult, owing to differences in ovarian cycles and responses to administered hormones in the donor and the recipient.) In examining the effects of oocyte cryopreservation, Tachibana et al. (2013) again used rhesus macaque oocytes. They found that while the oocytes tolerated cryopreservation well, cryopreservation negatively impacted blastocyst development, with only 6% of the fertilized oocytes developing into blastocysts. Reciprocal spindle-chromosomal complex transfer was then conducted between fresh and previously frozen oocytes. It was found that when fresh spindles were transferred into the frozen-thawed cytoplasts, fertilization and blastocyst development were impaired. When frozen-thawed spindles were transferred into fresh cytoplasts, however, fertilization rates and blastocyst development were comparable to the fresh controls. The cryopreservation process, therefore, appears to damage primarily the cytoplasm and not the spindle complex. The blastocysts derived from fresh cytoplasts and frozen spindles were then developed into two embryonic stem cell lines and transplanted into a rhesus macaque recipient, successfully resulting in pregnancy and birth (Tachibana et al. 2013). This process, although conducted in monkeys, is very promising for spindle-chromosomal transfer in humans.

3.1.3 Polar body genome transfer

A new method of using polar bodies in MRT has been proposed. It has been demonstrated that both polar bodies produced from meiosis contain the same genetic material as the primary oocyte and are able to develop normally (Wakayama et al. 2007). The advantages cited by Wang et al. (2014) of using polar bodies are that polar bodies consist mainly of chromatids, and contain comparatively few organelles (notably, mitochondria). In addition, polar bodies are easily visualized, and their use may increase MRT efficiency, as comparatively few oocytes are required. Wang et al. (2014) conducted experiments using polar body transfer in mice. Their methods and results were as such:

1. Mitochondrial content was measured by immunofluorescence staining, which was used to visualize cells. The first polar body (PB1) contained far fewer mitochondria than the primary oocyte used in spindle-chromosomal transfer. The second polar body (PB2) also contained fewer mitochondria than the zygote used in pronuclear transfer. The formation of the spindle-chromosomal complex in the oocyte, and zygotic activation, both trigger mitochondrial aggregation (or the clumping together of mitochondria), which promotes carryover, whereas polar body genome transfer avoids mitochondrial aggregation.
2. Mitochondrial content was also measured by PCR to estimate the mtDNA copy number in germline karyoplasts. It was determined that PB1 contained fewer mitochondria than the spindle-chromosomal complex in the oocyte, and that PB2 contained fewer mitochondria than in the pronuclei of the zygote.
3. Using staining, PB1 and the oocyte were compared. It was found that they were similar in their cytoskeletons and nuclear integrity. Additionally, PB1 did not exhibit any differences from the oocyte in epigenetic markers.
4. PB1s were fused with enucleated donor oocytes for spindle-chromosomal complex transfer,

with high success. This demonstrated that using PB1s as a source of genetic information in spindle chromosomal-complex transfer could greatly increase the efficiency of MRT.

5. The membrane integrity, morphology, and nucleus sizes of PB2 and the female pronucleus were compared. They were found to be similar in all respects. When examining epigenetic markers that may affect gene function, PB2 and the female pronucleus were also found to be similar.
6. PB2 was fused with a half-enucleated zygote (with the second polar body replacing the female pronucleus), with high success. This demonstrated that PB2 could successfully be used in pronuclear transfer to increase the efficiency of MRT.
7. While the previous experiments were successful in terms of post-manipulation survival, it remained unknown whether the manipulated complexes could develop normally. The spindle-chromosomal complex transfer oocytes that used PB1 were fertilized, exhibiting cleavage rates and blastocyst development similar to the embryos that underwent normal spindle-chromosomal complex transfer. The zygotes that underwent pronuclear transfer using PB2 had cleavage rates similar to the zygotes that underwent normal pronuclear transfer, but lower rates of blastocyst development.
8. The manipulated blastocysts were transferred into pseudopregnant mothers to observe in vivo development. In the blastocysts containing PB1s and in the blastocysts containing PB2s, birth rates were comparable to the controls and all offspring were healthy.
9. The rate of mtDNA carryover was determined by pyrosequencing (which sequences the DNA and can identify mutations). The spindle-chromosomal complex transfer using PB1 yielded undetectable levels of heteroplasmy, and the pronuclear transfer using PB2 yielded a carryover rate of 1.7%. Both groups showed much lower heteroplasmy than either the simple spindle-chromosomal complex transfer group or the simple pronuclear transfer group.
10. Finally, the genomic integrity of the polar bodies was confirmed by array comparative genomic hybridization (aCGH), which compares chromosome copy numbers. As expected, PB1 and the spindle-chromosomal complex yielded the same results, and PB2 and the female pronucleus yielded the same results (Wang et al. 2014).

These experiments suggest that the use of polar bodies as a substitute for nuclear genetic material in previously established MRT methods yields favourable results. The inclusion of polar bodies in these processes results in normal offspring with markedly less mtDNA carryover. Additionally, both the PB1 and PB2 of a donor oocyte can be used in MRT, which reduces the number of oocytes used and thereby increases the overall efficiency of MRT. Polar body genome transfer has the potential to optimize both spindle-chromosomal complex transfer and pronuclear transfer for clinical use.

3.2 Criticism of techniques involved in mitochondrial replacement therapy

There are multiple concerns about the techniques involved in MRT. One main concern is the increased proportions of mutated mtDNA, while another regards the overall efficacy of MRT in preventing mtDNA mutations in successive generations.

Sato et al. (2005) conducted a successful rescue experiment in mice using pronuclear transfer. However, by monitoring mutated mtDNA in cells from the mouse's tail, they found that the proportions of mutated mtDNA increased over time. The increase in accumulated mutated mtDNA was 17% during gestation, 8% in the first 30 days after birth, another 8% in the following 100 days, and an additional 6% in every 100-day period after that point. Sato et al. (2005) attributed the reduced rate of increase to a decreased rate of cell division. They predicted that the proportion of mutated mtDNA would be 43% at day 800, indicating that the mito-mice would not die from mitochondrial disease, but rather from old age. Humans, however, have a much longer lifespan and thus more time to accumulate mutated mtDNA, which has negative implications for the use of MRT in humans (Sato et al. 2005). In similar experiments performed by Craven et al. (2010), a lower proportion of mutated mtDNA was found in the embryos: 8.1% the first time, and 1.7% the second time, when the researchers ensured that the amount of cytoplasm carried over from the donor was minimal (Craven et al. 2010). The spindle-chromosomal complex transfer experiments showed much lower mutated mtDNA carryover; however, small amounts were still present (Tachibana et al. 2009; Tachibana et al. 2013).

As a result of mtDNA carryover, and due to the bottleneck effect, MRT may not have the desired effect of eliminating the mutated mtDNA from the germline. Any remaining mutated mtDNA might be passed down to offspring in a higher proportion, resulting once again in the disease phenotype. While this therapy may be effective in one generation, it may not be effective in successive generations, raising the question of whether MRT is only a temporary solution. If it is only temporary, it may not be a useful therapy in which to invest a considerable amount of time and resources, considering the relative rarity of the diseases associated with mtDNA mutations (Baylis 2013).

4. Current legal and ethical issues

In Canada, the Assisted Human Reproduction Act makes heritable manipulations to the genome or embryo illegal. This categorization includes MRT (Bill C-6 2004). In the United Kingdom, legislation was passed to make MRT legal, effective October 29th, 2015 (Parliament of the United Kingdom 2015). In the United States, the National Academies of Sciences, Engineering and Medicine, appointed by the Food and Drug Administration (FDA) to review MRT, released a report in February of 2016. This report recommended the approval of MRT in the United States, advising that human clinical trials initially be performed with only male embryos (National Academies of Sciences, Engineering, and Medicine 2016). The reason underlying this cautious approach is that the effects of MRT will be observable in male offspring, but the manipulated mtDNA will not be inherited by future generations, owing to the matrilineal pattern of mitochondrial inheritance. However, despite the recommendation of the National Academies of Sciences, Engineering, and Medicine, legislation has not allowed clinical trials to begin in the United States (114th Congress, 2016).

Moreover, bioethicist Françoise Baylis (2013) raised concerns about numerous aspects and implications of MRT. One such concern involved the wellbeing of the egg provider. Baylis raised the excellent point that when women go through the uncomfortable medical side effects of the IVF process, the benefit of becoming pregnant with a biological child often outweighs the costs. An egg provider is granted no such reward at the end of the procedure – only emotional and potentially financial benefits. The possibility of financial benefits for donors of healthy eggs also raises concerns about the possible coercion of egg providers, particularly among economically disadvantaged groups. Another ethical consideration is the wellbeing of the resultant offspring themselves. Baylis voiced concern about the success of the therapy, and whether it could unintentionally introduce new malignant effects in offspring. Therefore, the costs of this therapy must be weighed against the benefit of having genetically-related children, especially when there are available alternatives (such as adoption and egg donation) which do not pose a potential risk to a child. Baylis also raised the point that regardless of how little the third party genetically contributes to the child, the contribution would play a part in the child's personal identity. The final concern Baylis presents is about MRT's broader implications. She expressed apprehension over the possibility of MRT being used for non-therapeutic means, and acting as a gateway to further editing of the germline (potentially leading to "designer babies"). As previously noted, Baylis also questioned the overall value of MRT research, and whether it is worth the time and money invested into it when there are conditions that affect a greater proportion of the population and have no treatment alternatives (Baylis 2013).

David King of Human Genetics Alert shared many of Baylis's concerns. In addition to these, he focused on the issue of preserving the integrity of the human being. He harboured the worry that by "cobbling together" various genetic contributions, MRT violates nature (King 2012).

Despite these concerns, the Nuffield Council on Bioethics ruled that MRT is an ethical procedure, given that it is thus far safe and effective. The Nuffield Council also stated that an egg donor should not be considered a third parent to the offspring, given the minute genetic contribution of mtDNA, despite the colloquial term "three parent baby" (Nuffield Council on Bioethics 2012).

5. Current and future avenues for mitochondrial replacement therapy

MRT has recently been employed in a clinical setting in Mexico, allowing a mother with Leigh syndrome to give birth to a healthy child (Canadian Broadcasting Corporation 2016). In future clinical use of MRT, prospective parents would begin with genetic counselling to confirm the mtDNA mutation. MRT would then be used as part of the IVF process to produce a healthy child. Future research should concern itself with optimizing the procedure to eliminate mtDNA carryover, to ensure that mutated mtDNA does not accumulate over an individual's lifespan, and that it is not passed on to future generations.

6. Conclusion

Currently, MRT is being developed to provide a solution for those who are at risk of passing on potentially harmful mtDNA mutations to their offspring. These techniques include pronuclear transfer (conducted in the zygote after fertilization) and spindle-chromosomal complex transfer (conducted in the oocyte before fertilization). Although both techniques have been successful in animal and human models, it appears that spindle-chromosomal complex transfer is more effective. Polar body genome transfer has also been researched as a means of further optimizing these MRT methods. To be sure, there are many legal and ethical issues surrounding this therapy. Nevertheless, substantial progress has been made in recent years to employ MRT in a clinical setting, where it has initially proven to be successful.

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