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Review

Therapeutic treatments of mtDNA diseases at the earliest stages of human development

Marcos Roberto Chiaratti [a,b], Flávio Vieira Meirelles [b], Dagan Wells [a], Joanna Poulton [a,*]

[a] Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford OX3 9DU, United Kingdom
[b] Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga 13635-900, Brazil

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A B S T R A C T

More than 150 pathogenic mitochondrial DNA (mtDNA) mutations associated with a range of illnesses have been described in humans. These mutations are carried by one in 400 people and their inheritance is exclusively maternal. Currently there is no method to prevent mtDNA diseases, which highlights the need for strategies to predict their transmission. Here we outline the scientific background and unique difficulties in understanding the transmission of mtDNA diseases, explaining why their management has lagged so far behind the genetics revolution. Moreover, both current and future management options, including cytoplasmic and nuclear transfer, are also discussed.

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1. Introduction

Pathogenic mitochondrial DNA (mtDNA) mutations are carried by one in 400 adults and children (Manwaring et al., 2007). Patients present, often in the neonatal period, with a range of devastating conditions including epilepsy and liver failure, cardiomyopathy and/or sudden death (van den Ouweland et al., 1992; Santorelli et al., 1994; Blok et al., 1997; Chinnery et al., 2000; Solano et al., 2001; Manwaring et al., 2007; Sacconi et al., 2008). More common are milder disorders such as age-related deafness (Manwaring et al., 2007) and/or diabetes (van den Ouweland et al., 1992), and loss of vision due to optic neuropathies (Wallace et al., 1988). Here, the scientific background and unique difficulties in understanding the transmission of mtDNA diseases are outlined, explaining why their management has lagged so far behind the so-called "genetics revolution" (Poulton and Turnbull, 2000). However, a significant step towards a therapeutic treatment, nuclear transfer, was reported in August 2009 when a "three parent monkey" was produced (Tachibana et al., 2009). This non-human primate model suggests that maternally inherited mtDNA diseases might now be clinically

* Corresponding author. Address: Women's Centre, Level 3, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom. Tel.: +44 01865 221004; fax: +44 01865 769141.
E-mail addresses: chiarattimr@gmail.com (M.R. Chiaratti), meirellf@usp.br (F.V. Meirelles), dagan.wells@obs-gyn.ox.ac.uk (D. Wells), joanna.poulton@obs-gyn.ox.ac.uk (J. Poulton).

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treatable at the outset of embryogenesis. Here, we describe both current and future management and treatment options, and why new studies in both human and monkey are so exciting as potential cures for metabolic diseases of mitochondrial origin.

The mitochondrion is a cytoplasmic organelle commonly referred to as the "power house" of the cell because of its role in oxidative energy metabolism. Most of the energy necessary for cellular functions is produced in mitochondria by oxidative phosphorylation (OXPHOS) and stored in the form of ATP, which supports most of the energy-dependent processes in cells (Wallace, 2005). Each mitochondrion has approximately 1500 polypeptide components of which most are encoded by nuclear DNA (nDNA). Nonetheless, of the over 80 polypeptides that are needed for OXPHOS, 13 are encoded in mtDNA. The mtDNA also encodes 22 unique tRNAs and two rRNAs involved in the translation of mitochondrial mRNA. Human mtDNA is a double-stranded (heavy and light strands), closed-circular molecule composed of 16,569 base pairs. Unlike nDNA, there are usually hundreds to thousands mtDNA copies in every cell, with the number of copies varying depending on cell type and metabolism. Cells of tissues with the highest energy requirements (i.e., neurons and muscle cells) contain more mtDNA molecules than those of other tissues such as leukocytes and monocytes (Miller et al., 2003; Casula et al., 2005). The mtDNA molecule is organized in nucleoprotein complexes known as nucleoids that are devoid of histones (Scarpulla, 2008a, 2008b).

According to the endosymbiotic theory of mitochondrial origin in eukaryotic cells, mitochondria are evolutionary descendents of an aerobic prokaryote. This theory proposes that mitochondria became specialized in energy production after symbiosis with an anaerobic cell, an event that probably occurred some 1.5 billion years ago. The remaining double membrane structure, circular genome and the presence of a complete set of prokaryotic-like tRNAs and rRNAs are "molecular fossils" that support this theory. During evolution, most of the proto-mitochondrial genes were apparently transferred to the nDNA. This includes the 70 polypeptides involved in OXPHOS as well as factors responsible for regulating replication, transcription and part of translation of the mtDNA (Smith et al., 2000; Wallace, 2005).

Since mitochondrial function depends on polypeptides encoded both by nDNA and mtDNA, nuclear-mitochondrial interactions are critically important to cellular health (Spelbrink et al., 2001). Hence, the expression of subunits encoded by both genomes must be closely coordinated. This is accomplished by a variety of well orchestrated regulatory mechanisms dependent on intracellular and extracellular signals including hormones and environmental stimuli (Garesse and Vallejo, 2001). Many factors are involved in coordinate nDNA and mtDNA expression, but the most known factor is the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1A), which is encoded by the nDNA. At the nuclear level, the PPARGC1A regulates expression of the nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which in turn coordinates (in the nucleus) expression of OXPHOS polypeptides and factors controlling replication, transcription and translation of the mtDNA. At the mitochondrial level, both replication and transcription of mtDNA are regulated by factors imported by mitochondria from the cytoplasm that interact with a specific region in the mtDNA known as Displacement loop or D-loop. Promoters of transcription of mtDNA heavy and light strands are located in this region, as are some of the mtDNA replication origins (Holt and Jones, 2009). Factors such as the mitochondrial transcription factor A (TFAM), transcription factors B1 and B2 (TFB1M and TFB2M) and a core RNA polymerase (POLRMT) encoded in the nDNA interact with the D-loop and are responsible for initiating bidirectional transcription of mtDNA. These factors also regulate mtDNA replication because the nascent RNA strand serves as a primer for initiation of mtDNA replication. Mitochondrial DNA replication also requires a specific DNA polymerase (POLG) (Scarpulla, 2008a, 2008b) as well as a helicase (specifically the Twinkle helicase which has most structural similarity to phage T7 gene 4 primase/helicase) (Spelbrink et al., 2001).

As well as depending upon these molecules for expression and maintenance of mtDNA, mitochondria require a group of proteins that enable them to split and fuse. Mitochondrial dynamics appear to be essential because defects in either splitting (Waterham et al., 2007) or joining can cause both disease (Alexander et al., 2000) and developmental anomalies (Chen et al., 2003). These processes enable the mitochondrial network to regulate energy supply within the cell and to maintain mitochondrial quality by recycling damaged mitochondria (Chen and Chan, 2005; Scherz-Shouval and Elazar, 2007).

2. Mitochondrial inheritance

Mitochondrial inheritance in mammals is uni-parental and maternal, since mitochondria in the offspring are inherited exclusively from the mother. This is because the oocyte contributes many more mitochondria (100,000 mitochondria compared to only 100) (Diez-Sanchez et al., 2003) and hence mtDNA to the zygote than the sperm, the few mitochondria that may persist in the newly fertilized oocyte are destroyed during early cleavage stages by ubiquitinylation and proteolysis (Sutovsky et al., 1999, 2000). Eliminating sperm mtDNA may be advantageous for reproductive success as it may be damaged by the high levels of reactive oxygen species (ROS) generated before fertilization (Smith et al., 2000). If mutations arising from oxidative damage were transmitted to the next generation they could compromise mitochondrial function leading to a dysfunction of the organelle and potential metabolic diseases (see below). Furthermore, clonality of maternal mtDNA is maintained by the "mitochondrial bottleneck" (Hauswirth and Laipis, 1982; Olivo et al., 1983; Ashley et al., 1989; Poulton, 1995; Poulton et al., 1998; Poulton and Marchington, 2002), whereby a small number of mtDNA genomes become the founders for the offspring. This may have evolved to minimize problems arising from the need for nucleo-mitochondrial interactions.

Another important feature of mitochondrial biology relates to the homogeneity of its genome within individuals. Thousands of mtDNA copies are present in every nucleated cell and normal individuals are homoplasmic, i.e. virtually all their mtDNA copies are identical (Koehler et al., 1991). However, individuals affected by mtDNA diseases are usually heteroplasmic, i.e., most of their tissues and cells have a mixture of both normal and mutant mtDNAs, with proportions varying between 0 and 99% (defined as the mutant load). There is also a threshold effect in most mitochondrially based metabolic diseases whereby tissues function normally unless the mutant load rises above a specific level. These thresholds vary with both tissue and mutation type from $<10\%$ to 100% mutant mtDNA (Sacconi et al., 2008; Poulton et al., 2010). Because the rate of nucleotide substitution in mtDNA is five to ten times faster than in nDNA (Brown et al., 1979, 1982; Parsons et al., 1997), mtDNA is highly polymorphic such that mutant and wild-type molecules coexist in a same cell or individual (heteroplasmy). Thus, one would expect to find much sequence variation among individuals and even more variation among species. However, coding sequences of the mtDNA have been preserved among species during evolution. Moreover, although differences exist in non-coding sequences of the mtDNA among individuals of the same species, it is rare to find heteroplasmic differences within a specific individual. Hence, the unexpected low frequency of heteroplasmy within a cell or individual contrasts with the frequency of mtDNA polymorphisms in the population at large (Cree et al., 2009; Poulton et al., 2010).

3. Mitochondrial DNA diseases: the bottleneck hypothesis and mtDNA transmission

Collectively, mitochondrial dysfunction underlies a group of human diseases whose severity ranges from mild to lethal. Because

mitochondria generate almost all cellular ATP, tissues affected by mitochondrial dysfunction(s) are commonly those whose energy demands are the highest, such as the nervous system, muscle and liver. Since mitochondrial function depends on the orchestrated communication between nDNA and mtDNA, mutations in either genome can lead to dysfunction. Even though it only encodes 13 respiratory chain components, population screening shows that about one in 400 people have a mutation in the mtDNA with phenotypic consequences (Manwaring et al., 2007). More than 150 pathogenic mtDNA mutations have been described in humans (Solano et al., 2001) that are associated with a range of severe to mild illnesses, including deafness, blindness, cardiomyopathy, liver failure, diabetes, and neuropathy via a number of perturbed cellular processes (Chinnery et al., 2000; Schaefer et al., 2004, 2008; Cree et al., 2009; Wallace and Fan, 2009).

Genetic management of patients with mtDNA diseases depends on understanding both germline segregation and on the physiological basis of the bottleneck (Poulton et al., 2010). However, while the available human data is minimal, statistical analysis of oocytes in human, mouse and *Drosophila* datasets shows that the distribution of mutant mtDNA in one case could be consistent with random drift, but does not exclude the possibility of selection in the germline (Elliott et al., 2008; Fan et al., 2008; Stewart et al., 2008; Wonnapinij et al., 2008; Cree et al., 2009; Wallace and Fan, 2009). In another family, a new pathogenic mutation appeared to be absent from the mother suggesting that it arose de novo within the germline, consistent with the high variance in the level of mutant mtDNA in her oocytes (Marchington et al., 2009).

In a classic study, Hauswirth and Laipis (1982) analysed a heteroplasmic D-loop sequence variant in Holstein cows and documented rapid switching of one homoplasmic clone to complete homoplasmy for another within a few generations. In humans, a similar pattern of rapid segregation has been reported in families affected by mtDNA diseases (Vilkki et al., 1990; Santorelli et al., 1994; Ghosh et al., 1996; Blok et al., 1997; Elliott et al., 2008). How polymorphic mtDNAs segregate and reestablish homoplasmy so rapidly remains unclear. Hauswirth and Laipis proposed that a reduction in the mtDNA content and subsequent clonal proliferation of this small group of mtDNA molecules during oocyte growth could account for the rapid shifts in mtDNA genotype frequencies between generations (Hauswirth and Laipis, 1982; Michaels et al., 1982). This has been termed the mitochondrial bottleneck. Later, the same research group proposed a second mechanism that also contributes to this switching (Olivo et al., 1983; Ashley et al., 1989). Mitochondrial DNA barely replicates during the early stages of embryo development and pre-existing mtDNA molecules segregate among the cells of the blastocyst (Piko and Taylor, 1987; McConnell and Petrie, 2004; Thundathil et al., 2005; Cree et al., 2008). The inner cell mass at this stage comprises a very few cells, yet it will ultimately give rise to the entire embryo. Hence both clonal proliferation of mtDNA in the developing oocyte and mtDNA segregation into a few cells of the blastocyst could underlie the bottleneck that selects only a small number of mtDNAs to colonize the next generation. This has given rise to the concept of a segregating unit, or a group of clonal mtDNAs that co-segregate. If the number of segregating units that become the mtDNA founders of the embryo is small, then large switches may occur in a single generation.

The hypotheses concerning the molecular mechanism(s) underlying the bottleneck were investigated by Jenuth et al. (1996) in lines of heteroplasmic mice. The authors concluded that the main component of the bottleneck was random genetic drift occurring during oogenesis, and that this could explain the rapid segregation of mtDNA variants. They measured the genotypic variance in primordial germ cells (PGCs) and found that it is markedly less than that of primary oocytes and mature oocytes. Thus, a sharp reduction in the mtDNA content of the cell, to perhaps ~200 molecules in PGCs, is followed by a large expansion in the oogonial population, thus explaining the patterns of mtDNA segregation that result in rapid shifts in mtDNA genotype frequencies between generations. Recent studies have carefully quantified mtDNA copy number of individual cells during development (Cao et al., 2007; Cree et al., 2008; Cao et al., 2009). Some evidence suggests that the relatively small number of mtDNAs in developing germ cells and during early in oogenesis corresponds to the number of segregating units inferred from post-natal analysis (Cree et al., 2008). However, this is controversial as it depends on technically demanding measurements of the number of mtDNA in single cells (Cao et al., 2007; Cao et al., 2009).

A mathematical simulation suggests that 70% of the mtDNA genotypic variation seen between two generations in mice is explained by partitioning of mtDNA molecules into daughter cells before mtDNA replication is reestablished in PGCs (Cree et al., 2008). The remaining 30% of variation was credited to the intense proliferation of the mtDNA during PGC expansion (Cree et al., 2008). Nonetheless, Wai et al. (2008) revisited their earlier analysis of mtDNA copy number and heteroplasmy during pre- and post-implantation development and concluded that these two processes (Cree et al., 2008) cannot account for the shifts in mtDNA genotype frequency. Indeed, Wai et al. (2008) identified a replicating subpopulation of mtDNA molecules during folliculogenesis by BrdU labeling, and the findings were in line with the earlier predictions of Hauswirth and Laipis (1982). This mechanism would (i) amplify a small pool of mtDNA molecules that would ultimately populate the oocyte and therefore found the next generation's mtDNA and (ii) could account for the rapid reestablishment of homoplasmy. Yet, this is still controversial largely because the small quantity of data available leaves room for substantial uncertainty (Samuels et al., 2010; Wonnapinij et al., 2010). Moreover, additional mechanisms appear to guide mitochondrial inheritance when the mtDNA is affected by variants in its coding sequences (see below).

4. Potential mechanisms that select against pathogenic mtDNA mutations in the germline

Mitochondrial DNA follows rules of inheritance that are distinct from the classical rules of Mendelian genetics. Pathologies caused by mutations in mtDNA will be inherited exclusively from the mother, and transmission to descendents will depend on the selection mechanisms described above. In non-human studies, these mechanisms efficiently reestablish homoplasmy within a few generations (Hauswirth and Laipis, 1982; Olivo et al., 1983; Laipis et al., 1988; Ashley et al., 1989; Koehler et al., 1991; Meirelles and Smith, 1997). Such mechanisms might ensure that pathological mtDNA mutations are not transmitted through many generations because the homoplasmic offspring then undergo selection at the level of the individual. If the mtDNA mutation severely compromises mitochondrial function, the individual will die and the mutation will never be transmitted. Three recent reports have suggested that there may be a mechanism that actively selects against pathogenic mtDNA mutations in the mouse. In a mouse model of mtDNA rearrangements the level of mutant mtDNAs in a mother's oocytes fell with time (Sato et al., 2007). According to Fan et al. (2008), selection against an mtDNA mutation that appeared to be severe was more effective than selection against another that was mild. As in the study by Sato et al. (2007), selection seemed to have occurred by the time oocytes were mature. At last, Stewart et al. (2008) have demonstrated negative selection against multiple mutations within mtDNA coding sequences through several generations in mice. One of the possible mechanisms proposed to explain these findings is selection at the organellar level during early stages of development (Shoubridge and Wai, 2008). During oogenesis/folliculogenesis mitochondrial division appears to outstrip mtDNA replication, diluting mtDNA to about one copy of mtDNA per organelle (Michaels et al., 1982; Jansen and de Boer, 1998;

Cao et al., 2007). Mitophagy is an intracellular mechanism for recycling cellular components that targets damaged mitochondria. As the phenotype of such an organelle would thus to be determined by only one mtDNA molecule, detrimental mutants might thus be removed from the next generation by this process (Scherz-Shouval and Elazar, 2007; Shoubridge and Wai, 2007). In addition, recent evidence suggests another mechanism for selecting the "best mtDNAs", based on a structure is known as Balbiani body (Kloc et al., 2004; Pepling et al., 2007). The Balbiani body is typical of early oogenesis, and it is comprised of mitochondria and endoplasmic reticulum organized around Golgi elements (Kloc and Etkin, 1995; Kloc et al., 1996; Kloc and Etkin, 1998; Kloc et al., 2004; Pepling et al., 2007; Zhang et al., 2008). This structure appears to enable germ plasm mRNAs to be specifically inherited by the PGCs in the future embryo. In the same way, a specific mitochondrial sub-population may segregate to the Balbiani bodies and ultimately populate the PGCs (D'Herde et al., 1995; Cox and Spradling, 2003; Kloc et al., 2004; Cox and Spradling, 2006). Selection of these mitochondria for the Balbiani body on the basis of function could explain reported patterns of mitochondrial inheritance. Such a mechanism may well occur in some non-mammalian species where mitochondria with the highest membrane potentials are found in Balbiani bodies (Cox and Spradling, 2003; 2006; Zhang et al., 2008), but this notion is controversial for the mouse (Wai et al., 2008).

5. Methods to predict and prevent transmission of mtDNA diseases

Heteroplasmy of mtDNA in the products of conception has important implications for all types of pre-natal diagnosis of mtDNA diseases. Couples who have lost a child due to an mtDNA disease often seek genetic counseling before trying to conceive again. Despite understanding how mutations in the mtDNA can affect mitochondrial activity and tissue function, and how mtDNA diseases are inherited, we are still unable to accurately predict the risk of a woman affected by a mutant mtDNA transmitting her disorder to her descendents (Cree et al., 2009; Poulton et al., 2010). Oocyte donation would avoid all the problems associated with the presence of mutant mtDNA, but there is a shortage of oocyte donors. Donors cannot be recruited from among first-degree relatives: the mother's sisters could be carriers of mutant mtDNA and the father's sisters would be consanguineous (Poulton et al., 2010). However, many parents desperately want children who are genetically related to them.

Sampling and analysis of human oocytes has occasionally been used to predict the risk of recurrence of some mtDNA disorders (Marchington et al., 2009). This involves measuring the mutant load in 10-to-20 unfertilized oocytes obtained by conventional in-vitro fertilization (IVF) methods, i.e. ovarian stimulation and trans-vaginal oocyte aspiration. Oocytes are retrieved from the mother of the affected child and the mutant mtDNA assessed in each oocyte. The disadvantage of this approach is that the number of oocytes retrieved may be insufficient for a useful statistical evaluation to be undertaken. Furthermore, the oocytes are destroyed in the process of testing and consequently those found to have low, non-pathogenic, levels of mutant mtDNAs cannot be used for fertilization and implantation (Poulton et al., 2010). An alternative in this case would be the use of pre-implantation (PGD) or pre-natal genetic diagnosis (PND). Unlike oocyte sampling described above, in both PGD and PND, only a small fraction of the embryo or the placenta is sampled to determine the mutant load. The mutant load in the embryo or fetus is used to estimate the risk of the individual developing symptoms of an mtDNA disorder post-natally (Poulton et al., 2010).

Pre-implantation genetic diagnosis is often used to detect mitochondrial diseases caused by mutations in the nDNA and therefore inherited according to Mendelian's laws. However, in the case of diseases caused by mutations in the mtDNA, there is little data to confirm its reliability. As the distribution of mutant mtDNA can vary between cells and tissues (Jenuth et al., 1997; Aiken et al., 2008), it is not certain that the mtDNA mutant load within a sample of chorionic villus (~10 mg) reflects that of the fetus (Poulton et al., 2010). High or low levels of mutant mtDNA in tissue obtained by chorionic villus sampling (CVS) indicate the fetus is probably affected or unaffected, respectively (Bouchet et al., 2006). However, finding an intermediate level is unhelpful as it is currently impossible to predict how the proportion of pathological and normal mtDNA levels will change over time (Cree et al., 2009; Poulton et al., 2010). Moreover, even if prediction of the final mtDNA level was possible, the correlation between mutant dose and disease severity is often poor (Cree et al., 2009; Poulton et al., 2010). Thus, PND that samples chorionic villus cannot provide an absolute prediction of the mutant load in the fetus. The mtDNA disease that has proven most amenable to PND is neurogenic weakness, ataxia and retinitis pigmentosa (NARP), due to either the mtDNA 8993T>G or T>C mutations. In both cases, the mutant load and disease severity are strongly correlated (White et al., 1999). Hence, a low mutant load indicates the fetus has low risk of developing the disorder after birth.

An alternative to PND using CVS is PGD, an approach that involves the generation of embryos using IVF techniques. In most cases, one or two cells are biopsied from each of the embryos produced and tested to determine the mtDNA mutant load. Only embryos found to have low levels of mutant mtDNAs are transferred to the uterus and consequently any pregnancy established should be unaffected by mitochondrial disease (Fig. 1). Although existing data is somewhat limited, studies suggest that PGD reliably predicts the mutant mtDNA load in the embryo and therefore the risk that the baby will develop the mitochondrial disorder during his life (Thorburn and Dahl, 2001; Dean et al., 2003; Brown et al., 2006; Jacobs et al., 2006; Poulton et al., 2010). Moreover, recent reports showing selection against mutant mtDNA causing severe disorders (Fan et al., 2008; Stewart et al., 2008) have encouraged the use of PGD since the probability of finding a low risk embryo is high. For PGD, cells are usually biopsied three days after fertilization, at which time the embryos are typically at the 6-to-10 cell stage. Since mtDNA replication probably does not resume at this stage (Piko and Taylor, 1987; McConnell and Petrie, 2004; Thundathil et al., 2005; Cree et al., 2008), analysis of one blastomere is reasonably representative of the whole embryo (Dean et al., 2003).

PGD is performed after controlled ovarian hyperstimulation, which produces multiple oocytes that are then fertilized in vitro. This process usually yields several embryos, which may be sampled simultaneously, increasing the probability that at least one embryo with a low load of mutant mtDNA will be identified (Steffann et al., 2006). As only unaffected embryos are transferred, termination of pregnancies with high levels of mutant mtDNA becomes unnecessary. Although PGD has been successfully employed for the diagnosis of more than 100 different genetic disorders, the technology is not without limitations. The most significant drawback of PGD is that it depends upon IVF treatment, which cannot guarantee that embryos transferred to the uterus will implant and develop to term. However, the probability of a patient becoming pregnant after transfer of unaffected embryos is approximately 51% (Gutiérrez-Mateo et al., 2008). While such outcomes present a reasonable expectation of success, some patients may require several treatment cycles to achieve an unaffected pregnancy.

An alternative to biopsy of cleavage stage (day three) embryos is to remove trophectodermal cells two days later, at the blastocyst stage (day five). Blastocyst stage embryos are generally associated with higher IVF pregnancy rates and also have the advantage that several cells may be safely removed, leading to more accurate PGD results. However, the biopsy and analysis of blastocysts for the diagnosis of mtDNA disorders have not been attempted at this time, primarily due to concerns that the mutant load may vary between the cells sampled and the inner cell mass from which the fetus develops, is not sampled.

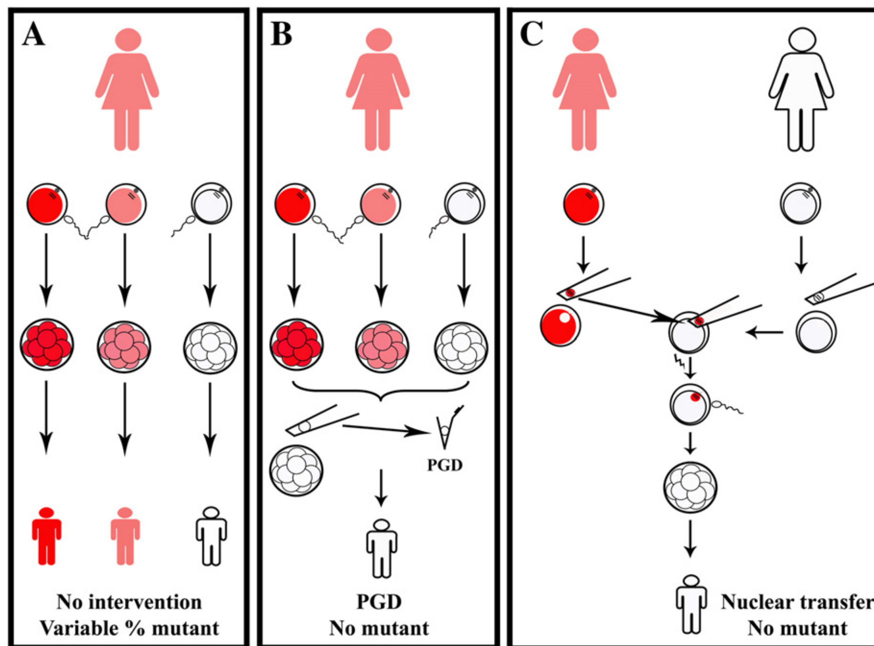

Fig. 1. Two possible ways to reduce transmission risks of mitochondrial DNA (mtDNA) diseases: pre-implantation genetic diagnosis (PGD) or potentially nuclear transfer. Red, pink and white colors represent high level, mid level and no mutant mtDNA, respectively. Panel A: if there is no intervention a carrier mother will have children whose mutant mtDNA load varies considerably. Panel B: pre-implantation genetic diagnosis involves sampling embryos (one cell sampling) that have been fertilized in vitro to estimate the load of mutant mtDNA. Only low risk embryos are replaced in the mother's uterus, and in the best case (illustrated) the mutant is not detectable in the offspring. This is becoming available in the United Kingdom for certain mtDNA diseases. Panel C: in nuclear transfer the carrier mother's oocytes (red color) are fertilized in vitro and, the nucleus removed and injected into donated oocytes (white color) that have been enucleated. In the best case (illustrated) the mutant is not detectable in the offspring.

Additionally, it is vital that PGD cases are followed-up to confirm the accuracy of the diagnostic strategies employed.

In spite of the benefits provided by PND and PGD strategies, they are unable to provide an absolute guarantee that offspring will not develop symptoms of the disorder and their validity is extremely limited in cases where mtDNA mutation load is poorly correlated with disease severity. Alternative strategies, which seek to treat rather than diagnose mtDNA disorders, include nuclear and cytoplasmic transfer (see below).

6. Cytoplasmic transfer

Cytoplasmic transfer (also called ooplasmic transfer) was introduced in humans in the late 1990s with the aim of treating infertile patients exhibiting persisting poor embryonic development and recurrent implantation failure (Cohen et al., 1997, 1998; Huang et al., 1999; Lanzendorf et al., 1999; Dale et al., 2001; Hwang et al., 2002). It had been proposed that such problems might be a consequence of depleted ATP levels or deficiency of other cytoplasmic factors (Van Blerkom et al., 1995, 2001; Chiaratti et al., in press). During cytoplasmic transfer, 5–15% of the cytoplasm from a presumptively viable oocyte is injected into the cytoplasm of an oocyte suspected of being compromised in some way, based on previous patient history with IVF. Not just mitochondria, but cytoplasm, RNAs, proteins, associated structures and energetic substrates from the donor oocyte are introduced into the recipient by this procedure. Hence it was expected that cytoplasmic transfer might correct the apparent deficiencies of the recipient oocyte, if the presumed embryo dysfunction indeed had a cytoplasmic etiology that could be addressed by replacing such a small volume of cytoplasm.

When cytoplasmic transfer was introduced into clinical practice at one centre and for a brief period, it did appear to rescue early embryonic development in some cases, enabling the birth of sixteen children (Barritt et al., 2001). As expected, the procedure results in progeny with mtDNA potentially derived from two sources: mother and cytoplasmic donor (Brenner et al., 2000; Barritt et al., 2001; Brenner et al., 2004). This had ethical implications because there are three genetic parents (Bredenoord et al., 2008) and some considered it as a type of gene therapy. Mitochondrial DNA analysis of live children showed that the contribution of donor mtDNA is small (Barritt et al., 2000; Brenner et al., 2000; Barritt et al., 2001), but in some cases, the proportion of donor mtDNA far exceeded the expected 5–15% proportion of extra cytoplasm injected (Brenner et al., 2004). Since heteroplasmy is a condition that is not commonly found in normal individuals, but is observed in patients with mtDNA-encoded disease, this generated great concern about the use of this technique in humans (Hawes et al., 2002; Malter and Cohen, 2002; Brenner et al., 2004; Sills et al., 2004).

The cytoplasm of an oocyte is highly structured (Fulton, 1993; Palermo et al., 1997; Levy et al., 2004; Sun et al., 2005), thus subtle problems might well arise if the maturation of the oocyte from which cytoplasm is transferred is not in perfect synchrony with the recipient oocyte. For instance, two human conceptions achieved following cytoplasm donation were found to be chromosomally abnormal (Barritt et al., 2001). Although the number of pregnancies achieved following cytoplasm donation is too small for any meaningful statistical analysis, the fact that two out of thirteen concepts were aneuploid generated concern over the safety of the method (Sills et al., 2004). Furthermore, one child developed a pervasive developmental syndrome at the age of eighteen months (Barritt et al., 2001). Thus, the question over the safety of cytoplasmic transfer remains unresolved at this time. However, it is currently known that chromosomal abnormalities occur at a high frequency in human oocytes mainly due to mis-segregation errors during the meiotic division (Holt and Jones, 2009). Thus, the chromosomal abnormalities seen in concepts born following cytoplasmic transfer may not be caused by the manipulation procedure itself, but due to factors intrinsically related to the patients (i.e. mother's age). This is further supported by the finding that the manipulation procedure needed for intra-cytoplasmic sperm injection (ICSI) is not far different than that

performed in the past for cytoplasmic injection and has not been associated with a much higher levels of miscarriage and birth defects compared to IVF (American Society for Reproductive Medicine, ASRM Practice Committee, 2008).

The use of cytoplasmic transfer for the purpose of correcting an mtDNA disorder has not yet been attempted, but the potential of this method to provide a cure, or at least an attenuation of symptoms, has generated some interest from researchers within the field (Thorburn and Dahl, 2001; Brown et al., 2006; Jacobs et al., 2006; Ferreira et al., 2010). Theoretically, cytoplasm donated by oocytes of healthy women could be used to dilute the mutant mtDNA level to below the critical threshold for disease; although a number of technical hurdles and questions of safety would need to be addressed prior to clinical application. To guarantee that offspring will not develop symptoms of the mitochondrial disorder, the mutant mtDNA level should be diluted to a very low level. This is particularly important in diseases where the critical threshold of mutant mtDNA is low (Sacconi et al., 2008). Only 5–15% of cytoplasm was introduced in the early cytoplasmic transfers, and this might be insufficient to lower the mutant load sufficiently. It might be possible to introduce a much larger amount of cytoplasm into the oocyte or to replace a proportion of the oocyte's cytoplasm. A possible way to do that would be by introducing purified mitochondria instead of cytoplasm. This has been performed using mitochondria isolated from somatic and embryonic cells but it does not seem to be enough to achieve high levels of donor mtDNA in the reconstructed oocyte (Van Blerkom et al, 1998; El Shourbagy et al., 2006; Hua et al., 2007). This might be overcome by partial depletion of mitochondria from the recipient oocyte/zygote following a procedure previously reported (Chiaratti et al., 2010; Ferreira et al., 2010). Nonetheless, the genetic contribution to the embryo is hard to predict because mtDNA from somatic and germline cells apparently behaves differently in the early embryo (Ferreira et al., 2007). Alternative methods that favor donor mtDNA replication over that of mutant forms have also been studied (Ferreira et al., 2010; Taylor et al., 2000). However, because of concerns about the safety of cytoplasmic transfer itself, in many countries the regulatory authorities have forbidden its use worldwide (Shoubridge, 2009). Therefore, before cytoplasmic transfer or mitochondrially-enriched cytoplasmic transfer becomes available as a therapeutic treatment, these concerns must be fully addressed using suitable animal models.

7. Nuclear transfer

The complete replacement of mutant mtDNA in a patient's oocytes with wild-type mtDNA would be the most radical method of reducing mutant load to avoid recurrence of mtDNA disorders (Poulton et al., 2010; Tachibana et al., 2009; Craven et al., 2010). Nuclear transfer enables an almost complete exchange of mtDNAs between two cells. This procedure involves removing the nucleus of a donor cell and transplanting it into a new one that had been previously enucleated. Since the latter will provide all cytoplasmic components, including mitochondria, virtually all mutant mtDNA molecules present in the nuclear-donor cell would be exchanged for wild-type ones. Hence, nuclear transfer is a potential tool for preventing the inheritance of mtDNA diseases (Fig. 1). As with cytoplasmic donation, an infant generated by this procedure will have three genetic parents: the couple who have donated the nDNA and a third person who has donated wild-type mtDNAs. However, because wild-type mtDNAs encode only non-mutant polypeptides needed to assemble the OXPHOS pathway, the infant will hopefully be free from risk of developing mtDNA disorders and will not inherit any physical or other characteristics from the mtDNA-donor individual.

Nuclear transfer has been performed successfully in mammals for over 20 years (Illmensee and Hoppe, 1981; Surani et al., 1984; Prather et al., 1987; Robl et al., 1987; Smith and Wilmut, 1989), but not until the 1990s was it used as a model to study mtDNA inheritance (Plante et al., 1992; Smith and Alcivar, 1993). Thereafter, many groups have developed diverse techniques to achieve nuclear transfer and most confirm that offspring inherit little if any nuclear-donor mtDNA (Steinborn et al., 1998; Evans et al., 1999; Meirelles et al., 2001; Hiendleder et al., 2003, 2005; Sato et al., 2005; Ferreira et al., 2007). Recently, two important steps have been taken towards using nuclear transfer as a tool to prevent inheritance of mtDNA diseases in humans. The first instance involved spindle-chromosomal complex transfer using non-human primate oocytes (Tachibana et al., 2009). The group responsible for this work removed the chromosomes from primate oocytes without significant mtDNA carry-over and transplanted into enucleated oocytes donated by another female primate. Tachibana et al. (2009) used mature oocytes arrested at metaphase II of the meiotic division because at this stage, the nucleus is almost devoided of surrounding mitochondria thus preventing introduction of a significant amount of mtDNA into the new embryo. Fertilization of oocytes after spindle transfer was successful, leading to the birth of primates that contained less than 3% of nuclear-donor mtDNA in all tissues analysed (Tachibana et al., 2009). It is highly likely that such a reduction in mutant load would be sufficient to prevent most mtDNA diseases in humans, suggesting that spindle–chromosome complex transfer would work as a therapeutic tool for mtDNA disorders. To date, the lowest threshold for a mutant mtDNA known to cause disease in humans is about 4–8% in cell culture, or levels of 25% mutant mtDNA in affected tissues (Sacconi et al., 2008). Thus, this procedure would have enormous implications for managing the inheritance of mtDNA disease after its safety in humans had been demonstrated. Moreover, Craven et al. (2010) have recently shown the feasibility of pro-nuclear transfer in humans, which resulted in minimal carry-over of nuclear donor mtDNA as the work by Tachibana et al. (2009), giving further support for the use of such methods to prevent transmission of mtDNA diseases. Different from spindle–chromosome complex transfer, in pro-nuclear transfer fertilized oocytes (at the pro-nuclear stage) are used as nuclear and cytoplasmic donors. Since at this stage the nuclear material is enveloped by a membrane, it minimizes the risk, compared to spindle transfer, of losing a chromosome during transplantation into the recipient cell. Nonetheless, the levels of nuclear-donor mtDNA were very variable among blastomeres of a single embryo, suggesting that mtDNA segregation might be disturbed by the procedure of Craven et al. (2010).

Another possible method of nuclear transfer suggested to be used as a tool for managing inheritance of mtDNA disease is germinal vesicle-stage karyoplast transfer (Brown et al., 2006). This procedure refers to transplantation of the genetic material between immature oocytes, which is possible to be carried out because chromosomes are enclosed in a clearly visible germinal vesicle. Although the presence of such vesicle makes transplantation safer and easier, the need for in vitro maturation and fertilization after transplantation limit its efficacy (i.e. currently there is no efficient method for in vitro maturation of human oocytes) (Chian et al., 2004).

Many questions related to the safety of nuclear transfer in humans must be answered before it becomes available clinically (Poulton et al., 2006; Shoubridge, 2009; Poulton et al., 2010). For instance, would the transferred nuclear material be able to regulate cytoplasmic-donor mtDNA? A complex interaction exists between nDNA and mtDNA that is not completely understood at this time. This issue must be fully investigated, especially given that subtle differences in maturational stage of the transferred nucleus and the recipient enucleated oocyte could lead to incompatibility and signaling miscommunication (Hawes et al., 2002; Malter and Cohen, 2002; Sills et al., 2004; Trounce and Pinkert, 2007). Apart from mitochondria, the recipient enucleated oocyte also carries other organelles and molecules that may induce epigenetic alterations of the nuclear genome, the consequences of which to the newborn are unknown (Hawes et al., 2002; Malter and Cohen, 2002; Sills et al., 2004; Poulton et al., 2010).

Safety issues notwithstanding, one of the greatest difficulties facing the introduction of nuclear transfer as a clinical therapy is the low efficiency of the technique (variable among germinal, spindle or pro-nuclear transfer). The probability of an oocyte that survives the manipulation procedure successfully fertilizing and producing a child is low, and therefore may require the use of many donated oocytes as cytoplasmic donors. In many countries, financial incentives for oocyte donation are not permitted and as a result the supply of donated oocytes is very limited. In countries where oocyte donors are paid, availability is better, but prices are high. The problems of price and availability will need to be overcome to make the use of nuclear transfer a viable option. Frozen oocytes or embryos set to be discarded might be an option as donors, but this depends on specific laws of different countries which need to be reviewed as well. Finally, PND and PGD may still be needed after nuclear transfer to check whether all nuclear material from the cytoplasm-donor cell has been exchanged by that donated by the nuclear-donor cell (i.e. to confirm euploidy). Additionally, although only few copies of mtDNA from the nuclear-donor cell are introduced into the oocyte or embryo, selective replication of these could occur leading to an increase in load of mutant mtDNA in the offspring or in specific tissues (Sills et al., 2004; Sato et al., 2005) that may thus lead to unintended consequence of introducing mtDNA disorder symptoms.

8. Conclusions

We conclude that genetic management of mtDNA diseases is improving and that PGD of mtDNA disease holds a great deal of promise, offering diagnostic advantages over traditional pre-natal methods of testing. Although therapeutic interventions for children and adults affected by mtDNA disorders remain extremely limited, there have been significant advances in the preconception correction of mtDNA disease (i.e. in oocytes), via cytoplasmic and nuclear transfer on experimental basis. However, at this time, such radical approaches to manage transmission of mutant mtDNA are forbidden in most countries. Before such methods can be considered as a viable approach to human disease, much additional work in model organisms, proving safety, efficacy, and improving efficiency, is required.

Conflict of interest statement

Oxford currently offers oocyte donation and oocyte sampling for mtDNA diseases. Preimplantation genetic diagnosis will be available in the near future. JP takes clinical and diagnostic referrals for the Oxford centre in the Rare Mitochondrial Disorders Service for Adults and Children (NCG). Further information may be found on http://www.obs-gyn.ox.ac.uk/research/jo-poulton.

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