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Mitochondrial DNA diseases: preimplantation diagnosis and intervention possibilities

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Mitochondrial DNA diseases: preimplantation diagnosis and intervention possibilities

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Index

List of Abbreviations

ATP: Adenosine Triphosphate

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CRISPR/Cas-9: Clustered Regularly Interspaced Short Palindromic Repeats - Associated Protein 9 System

DNA: Deoxyribonucleic Acid

GVT: Germinal-Vesicle Transfer

HFEA: Human Fertilisation and Embryology Authority

MST: Meiotic Spindle Transfer

mtDNA: Mitochondrial Deoxyribonucleic Acid

nDNA: Nuclear Deoxyribonucleic Acid

NT: Nuclear Transfer

PBT: Polar Body Transfer

PGD: Preimplantation Genetic Diagnosis

PNT: Pronuclear Transfer

Abstract

Mitochondrial DNA mutations are exclusively maternally inherited and can cause severe diseases for which there is no treatment and whose recurrence risk is difficult to estimate due to heteroplasmy and inheritance specificities such as the genetic bottleneck.

In this work we aim to review the scientific evidence on the current options for preimplantation genetic diagnosis and interventions to prevent these diseases.

Preimplantation genetic diagnosis can be performed through multiple techniques in different developmental stages of the oocyte or the zygote. Preimplantation genetic interventions rely on nuclear transfer, a set of different techniques in which the patient's nuclear genetic material is placed in an enucleated donor's cell, or on genomic edition, through which the mitochondrial genome is changed. All these methods are associated with several technical barriers. When applying preimplantation genetic diagnosis, the representativeness of the sample obtained must be confirmed. Incompatibilities between mitochondrial and nuclear DNA must be excluded when nuclear transfer is to be used. When genome edition is the choice, the absence of off-target modifications must be ensured. Ethical issues are also extremely relevant due to the possible germline modification or the selection of male embryos to avoid further transmission of residual mutated mitochondrial DNA.

Although much has already been accomplished, further research is required to clarify issues related to these techniques and also to develop more efficient and safe methods. All these developments will have to deal with a balance between scientific progress and ethical concerns.

Keywords: DNA, Mitochondrial; Mitochondrial Diseases; Preimplantation Genetic Diagnosis; Reproductive Techniques, Assisted; Gene Editing.

Resumo

As mutações do ADN mitocondrial são de herança exclusivamente materna e podem causar doenças graves para as quais não existe tratamento e cujo risco de recorrência é difícil estimar devido à heteroplasmia e a especificidades de hereditariedade como o *genetic bottleneck*.

Neste trabalho pretendemos rever a evidência cientifica sobre as atuais opções para diagnóstico genético e intervenção pré-implantatória para prevenir estas doenças.

O diagnóstico genético pré-implantatório pode ser realizado por múltiplas técnicas em diferentes estádios de desenvolvimento do oócito ou do zigoto. A intervenções genéticas pré-implantatórias agrupam-se em transferência nuclear, um conjunto de diferentes técnicas em que se coloca o material genético nuclear da doente numa célula doada enucleada, ou em edição genómica, em que o genoma mitocondrial é alterado. Todos estes métodos têm associadas várias barreiras técnicas. Quando o diagnóstico genético pré-implantatório é aplicado, a representatividade da amostra obtida deve ser confirmada. Incompatibilidades entre ADN mitocondrial e nuclear devem ser excluídas quando se usa transferência nuclear. Quando se opta por edição genómica, a ausência de modificações *off-target* deve ser assegurada. As questões éticas são também extremamente relevantes devido à possível modificação da linha germinativa ou à seleção de embriões do sexo masculino para evitar posterior transmissão de ADN mitocondrial mutado residual.

Apesar de tudo o que já foi alcançado, é necessária mais investigação para clarificar questões relacionadas com estas técnicas e ainda para desenvolver métodos mais eficientes e seguros. Todos estes desenvolvimentos terão de lidar com um equilíbrio entre o progresso científico e as preocupações éticas.

Palavras-chave: ADN mitocondrial, Doenças mitocondriais, Diagnóstico genético pré-implantatório, Reprodução medicamente assistida, Edição genómica.

Introduction

Mitochondria are cytoplasmic organelles commonly referred as the “power houses” of the cell because of their role in cellular bioenergetics by adenosine triphosphate (ATP) production.[1–4] This denomination may seem incomplete because mitochondria are responsible for many other essential functions, such as determination of cell death pathways,[2,5] calcium homeostasis [5,6] and formation of iron sulphur clusters.[6]

These organelles have some distinctive characteristics, one of the most important ones being the fact that, besides the regulation by the nuclear deoxyribonucleic acid (nDNA), mitochondria also have their own genome, the mitochondrial deoxyribonucleic acid (mtDNA). So far, more than 200 pathogenic mutations in mtDNA have been described,[2,7–9] which are responsible for 15% of mitochondrial disorders,[10–12] the most common group of inborn metabolic diseases,[13–15] and one of the most prevalent groups of neuromuscular disorders.[3] Although these diseases affect approximately 1/5000 people,[6,16,17] it is estimated that 1/400 [1,18–20] to 1/200 [2,6,8,21,22] people are carriers of these mutations. The exact prevalence of these mutations in the population is difficult to estimate without specific genetic studies [23] because of the high ratio of asymptomatic carriers.[2]

Mitochondrial diseases may be severely debilitating and even lethal as a result of decreased ATP production.[24] Due to this main pathogenic mechanism, there is preferential involvement of the most energy demanding tissues,[6,16,25,26] such as the central nervous system,[7,26,27] heart,[6,16,26,28] skeletal muscle,[6,16,26,28,29] liver,[16,26,28,29] kidney [26,28,29] and β cells of the pancreas.[7,29] These diseases can have their onset at any age [23,30] and usually have multisystemic or multiorgan involvement,[10,17,31,32] with multiple symptoms such as developmental delay,[14,20] epilepsy,[1,33] stroke,[8] deafness,[1,20,26] blindness, [1,20,26]

neuropathies,[20] diabetes,[1,20,26] myopathies,[2] loss of motor control,[14] renal failure,[26] liver failure,[1,20,26] exercise intolerance,[8,14,34] respiratory failure,[8] cardiomyopathies [1,20,33] and cardiac failure.[22,26] Abortion and stillbirths are also possible manifestations of mtDNA diseases.[14,15]

Considering their high frequency,[11] the severity of the phenotypes,[11,35] the absence of curative treatment,[11,17,21,32] and the high recurrence risk for offspring of female carriers,[11] transmission of these disorders should be prevented. The aim of this review is to describe methods that can be used to prevent the hereditary transmission of these disorders.

Materials and Methods

In order to perform this review of literature, we made our research on Pubmed using an equation combining the Mesh terms "DNA, Mitochondrial", "Mitochondrial Diseases", "Reproductive Techniques, Assisted" and "Preimplantation Diagnosis" and excluding the term "Infertility" and on Embase using an equivalent equation with the correspondent Emtree terms. We applied additional filters of Portuguese and English languages, of studies in Humans and of articles published in the last ten years and we excluded "Conference Abstract", "Letter" and "Editorial" typologies. In the end, we obtained and analysed 133 different articles in order to exclude those that did not match the topic of this work. Besides these articles, others relevant to the subject were occasionally considered.

Results

Mitochondrial DNA

MtDNA accounts for less than 0.1% of the total cell deoxyribonucleic acid (DNA).[33] It is a double-stranded circular molecule that encodes 37 genes, 13 of

which encode protein subunits involved in electron transport of oxidative phosphorylation, 22 encode transfer ribonucleic acids and 2 encode ribosomal ribonucleic acids, all of them involved in mitochondrial protein synthesis.[1,6,14,29,36]

MtDNA has some distinctive features. Each cell has between 500-10.000 copies of mtDNA (the actual number depends on cell type and metabolism),[1] except for the mature oocyte which contains 100.000-600.000 copies.[10,14,37] MtDNA mutations may be either homoplasmic (only mutated mtDNA is present in all tissues of an affected individual) [6,21,26,27] or, which is more common, heteroplasmic (there is a mixture of normal and mutant mtDNA, the proportion of mutated mtDNA varying among different tissues).[6,14,21,26]

In heteroplasmic conditions, clinical manifestations only occur when the mutated mtDNA load exceeds a threshold that is both tissue and mutation specific.[1,11,14] The mutation load and the severity of symptoms are predictably related,[20] although there is not always a genotype-phenotype correlation.[10,11,38,39] The proportion of mutated mtDNA can change over time because mtDNA can replicate in both dividing and non-dividing cells, which also contributes to the variable clinical severity of these disorders and their progressive nature.[19,20]

The inheritance of mtDNA is also distinctive because it does not follow the Mendelian patterns [24] as it is exclusively maternally transmitted,[1,16,17,40,41] because of the oocyte contribution with much more mitochondria than the spermatozoon (100.000 compared to only 100) [1,31] and because of the destruction of the few paternal mitochondria that may persist during early cleavage stages by ubiquitination and proteolysis.[1,42,43] Besides, the load of mutated mtDNA inherited by the fetus in heteroplasmic conditions is affected by the mitochondrial genetic

bottleneck,[6,11,16,17,26] a process through which a few mtDNA molecules become founders of the offspring.[18,20] This process is mutation and even individual specific [11,14,16] and causes unpredictable fluctuations in the proportions of mutated and normal mtDNA that are inherited.[6,38]

Methods to predict and prevent transmission of mtDNA diseases

1. Oocyte donation

Oocyte donation is considered the easiest way to prevent mtDNA diseases.[16,43] Although women who carry and give birth are recognized as the legal mothers,[10,14] they will not be genetically related to the child.[10,14,40] For this reason, this method will not be an option for some couples. One of the major problems with this method is the lack of oocyte donors.[1,10,14,16,19] In addition, using oocytes of close maternal relatives is not recommended because they can carry the same mutation.[1,10,14]

2. Oocyte testing

Oocyte testing may be used to estimate the recurrence risk after ovarian stimulation, collection of oocytes and analysis of their mutation loads.[1,20] Whenever the mutation load is below 5%, the option of natural conception may be acceptable, although there is no guarantee that the naturally selected oocyte will have a low mutation load. In order for this method to be a reliable option, a good correlation between mutation load and disease severity should exist.[20] This procedure has several disadvantages such as the requirement for ovarian stimulation, the low number of oocytes produced by some women and the inevitable destruction of these oocytes during the process of testing.[1,20,44]

3. Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) aims to transfer disease-free embryos after evaluation of their mutation loads as they may be different from the patient's, and may even differ between embryos, due to the genetic bottleneck and random segregation during oogenesis.[38,45,46] Analysing mtDNA is technically easier and less prone to artifacts than analysing nDNA due to the higher number of mtDNA copies per blastomere.[10,14]

There are three possible stages at which mitochondria may be accessed for evaluation: the first polar body, the blastomere and the blastocyst stage.[38]

The first polar body biopsy is performed before fertilization, which would be ethically acceptable for those that oppose to embryo testing.[38,47] However, it is still questionable whether the mutant load of the polar body is accurately representative of the mutant load of the oocyte.[38] In fact, recent studies showed a low correlation between them and it has been speculated that this may be due to the highly asymmetric segregation of mitochondria during female meiosis.[6,10,14,45,48]

Other available options are blastomere biopsy, performed at the 8-cell stage, at day 3, or blastocyst biopsy, at day 5.[1,16,18,38] In both techniques, one of the major challenges is the necessity to ensure the representativeness of the mutation load of the sample.[16,26,32,45,49]

As far as blastomere biopsy is concerned, there is no consensus whether one or two blastomeres should be used, because of the possible differences between their mutation load.[14] Some studies have shown that one blastomere may be representative in most cases [1,11,35] and it has the advantage of being less detrimental to the embryo's viability.[25,35,38] Nevertheless, as individual outliers exist, some authors recommend using two blastomeres and considering the higher percentage of mutated mtDNA when discrepancies are found.[10,14]

In blastocyst biopsies, trophectoderm cells are collected, as are judged to be representative of the inner cell mass of the embryo.[6,32,45] In this technique a greater amount of cells can be removed without a negative impact on embryo implantation and development potential,[14,45,49,50] allowing a more accurate prediction of the mutation load of the embryo [14] and reducing the risk of errors due to mosaicism.[45] Nevertheless, in this stage the cell to cell variation is higher and trophectoderm biopsy may be less reliable. Taking this into consideration, there is a trend to suggest that blastomere biopsy should continue to be preferred until more data are available.[35]

After analysing the embryo's mitochondria, it is recommended that embryos with a mutation load below a previously defined threshold should be transferred to the uterus.[11,14,16,20,40] This brings out another issue: the definition of the threshold level. The ideal option would be to only transfer embryos with no mutated mtDNA in order to completely eliminate the risk of disease transmission instead of only reducing it.[10,13,44,46,51] However, when the threshold is reduced, fewer embryos (or even none) will be available.[13,20,21,38] Defining an appropriate threshold is still difficult with regard to many mutations because of the lack of available data,[11,13,38,49] being almost impossible for rare or private mutations.[10,14] Considering this, studies have been performed to try to set a threshold that may be applied to all mtDNA mutations. 18% was the value obtained with 95% confidence,[10,13,14,32,40,52] but more evidence is still necessary to support this value.

Obtaining embryos with a mutation load below the defined threshold can require multiple ovulation induction cycles, being difficult to define the number of cycles that it may be acceptable to perform in order to find the best possible embryo.[10,14,44] In addition, embryos with the lowest mutation loads may not be the ones with better morphologic quality. In conclusion, more research is required in order

to find an adequate balance between mutation load and embryo morphologic quality and to identify the most appropriate embryos to transfer.[10,14]

It is also important to further investigate the mutation load variation through time.[10,14,16] Prenatal diagnosis is recommended to confirm its stability [10,20,38] and it is also advised to evaluate children that were conceived using this technique,[38,44] preferably through genetic testing as clinical symptoms may have a late onset.[44] Nevertheless, testing children can raise several ethical issues.[20,44,47]

Individual counselling is mandatory before using any of these techniques due to the uncertainties linked to them.[10,13,14,26,38] The discussion of the threshold to be applied should involve the couple because it depends on factors like the disease severity in the family, risk perception, the availability of embryos below the threshold and issues associated with *in-vitro* fertilization.[10,13,14,25]

Despite the existing experience with this technique that has been used since 2006, when it was first successfully accomplished, all this doubts still remain.[52]

PGD cannot be used in women with homoplasmic mutations [16,21,26,31,39,40] and has limited value for women with high mutation load, particularly if their oocytes carry a mutation load similar to their own,[16,26,40] and for women with mutations that have a poor correlation between mutation load and disease severity,[1,16] requiring further options.

4. Ooplasmic transfer

When it is not possible to select an embryo with a mutation load that is low enough for uterine transfer using PGD, different approaches are required to obtain embryos with adequate mutation loads.

A possible option is to perform ooplasmic transfer, also referred to as cytoplasmic transfer, which consists in the injection of ooplasm with normal

mitochondria from a healthy donor to an oocyte containing mutated mtDNA.[1,3,23,26,28,34,53]

It is believed that ooplasmic transfer would lead to a reduction of the effects of mtDNA mutations through a dilution effect,[23] but this is still a theoretical possibility, since it has not been used to prevent mtDNA disorders yet.[16,28]

One of the main barriers for the use of this technique is that only up to 15% of donor ooplasm can be transferred. To prevent the transmission of these diseases, a larger amount would be needed [1,16,23,28] and it is still unknown whether it is possible.[1,23,28] Alternative strategies would be the use of purified mitochondria instead of cytoplasm, or partially removing the mitochondria from the oocyte.[1,19] Another concern regarding this methodology is that, the long-term effects of introducing a new mtDNA haplotype into the oocyte are still poorly understood.[16,18] So far, multiple chromosomal abnormalities and birth defects were reported, leading to the prohibition of this technique worldwide.[1,3,16,53]

5. Nuclear transfer

Nuclear transfer (NT), also known as mitochondrial replacement therapy, is a set of techniques that consist in the removal of nDNA of the mutation carrier and its transfer to an enucleated oocyte from a mutation-free donor. Using these techniques, a new cell with nuclear genetic material from the patient and mtDNA from a donor is obtained, avoiding disease transmission.[4,14,18,23,26,31,54–57] It can be performed in oocytes or in zygotes through five different techniques: germinal-vesicle transfer (GVT), meiotic spindle transfer (MST), pronuclear transfer (PNT), polar body transfer (PBT), and blastomere transfer. While GVT and MST do not require fertilization, PNT and blastomere transfer can only be performed after fertilization. PBT depends on whether a first polar body or a second polar is used.

The United Kingdom was the first country to allow the use of these techniques in March 2015, when the Human Fertilisation and Embryology Authority (HFEA) approved the use of MST and PNT.[14,34,50] The HFEA considered these two techniques as potentially useful for patients whose offspring could have severe or lethal mtDNA diseases, and had no other option for having their own genetic children.[26,29,34,54,58,59]

There have always been concerns related to the fact that nuclear transfer techniques involve germline genetic modification,[10,23,26,29,50,60] and due to the erroneous idea of these children having 'three parents' or 'two mothers'.[3,7,17,18,23,61] In response to these questions, the Nuffield Council on Bioethics concluded that if these techniques are proven to be safe and effective, it would be ethical for families to use them due to the health and social benefits of a life free from mitochondrial disorders.[2,26,29,41] The Council also concluded that these techniques do not lead to a 'third parent' or 'second mother', neither biologically nor legally,[2,7,29,60] as the donor's genome contribution is just of 0.1%.[29]

A major concern linked to the use of these techniques is the co-transfer of mutated mtDNA bound to the nDNA.[14,15,20,21,26,31] Even if this occurs, low levels of mutated mtDNA would be expected, and usually not linked to disease manifestation.[14,26,29] However, there is still a slight risk of segregation of mutated mtDNA to specific tissues or the germline, with unpredictable effects for future generations.[3,21,26] Recent studies showed that the chance of disease recurrence in subsequent generations is reduced dramatically if a load of mutant mtDNA below 5% is achieved.[16,60] There are also concerns about one mtDNA haplotype replicating faster than the other,[16,22,43,62] enabling the transformed embryos to "revert" to a damaged condition.[58,63,64] This feature seems more likely to occur when large DNA sequence differences exist between haplotypes.[18,62] Thus, using oocytes with

similar haplotypes may minimize this risk, but further research is still required.[43,62] Due to these uncertainties, PGD may be required to confirm the low mutation load of the resulting embryo.[1,20]

Mismatch between nuclear and mitochondrial genomes also raises some concerns [2,6,14–16,22,26,63,65,66] because many of these genes have functional relations and depend on each other.[42] Some studies compared differentiation efficiency, mitochondrial enzymatic activity and the oxygen consumption rate between cell lines grouped based on single nucleotide polymorphism differences between the patient and the donor's oocyte mtDNA. Similar results were obtained, which suggests that compatibility between the nuclear genome of the patient and the donor's mitochondrial genome exists.[4,63] In fact, human outbreeding has induced extensive mtDNA and nDNA mixtures [53] and paternal genes are confronted with a potentially new mtDNA environment in each generation without adverse effects to the offspring.[67] Nevertheless, further investigation is advised. Until then, this might be another reason why using oocytes with similar haplotypes could be an advantage.[14,16,34,42,43,61]

Another concern is that epigenetic abnormalities such as Beckwith-Wiedemann Syndrome and Angelman Syndrome have already been associated to assisted reproductive technology.[26,65] Studies developed so far did not reveal any severe epigenetic changes following NT, but further research is still needed to confirm these results.[16,26,52,66]

In order to understand all the possible consequences linked to the use of NT, long-term follow up of children born using these techniques, and of future generations, is essential.[14,26,41]

At present, the main drawback for the clinical application of these techniques is their inefficiency and consequent requirement for many oocytes to generate one baby, when donated oocytes are scarce.[1,20]

NT, more specifically MST, has already been clinically applied with success in 2016, resulting in the birth of a male child with a reduced level of pathogenic mtDNA.[57,68]

a. Germinal-vesicle transfer

Germinal-vesicle transfer (GVT) consists in the transfer of nuclear genetic material from an immature oocyte at the germinal-vesicle stage into a previously enucleated donated oocyte. Fusion is obtained by electric pulses.[6,16,69] In this stage, mitochondria are concentrated in the peri-nuclear space and this may lead to co-transfer of a significant amount of the donor's mtDNA.[3,70]

Germinal-vesicle observation is easy and its removal is less invasive when compared to the other procedures.[28] Nevertheless, a major disadvantage of this procedure is the requirement of *in vitro* maturation of the oocytes,[1,6,16,28] which is still a suboptimal procedure [1,16,52] that leads to poor developmental competence of the oocytes.[69]

b. Meiotic spindle transfer

Meiotic spindle transfer (MST) involves removing the meiotic spindle from the mutation carrier's oocyte and placing it in a previously enucleated donor oocyte.[14,26,29,31,71]

The visualization of the spindle requires the use of liquid crystal birefringence and polarized light microscopy.[6,16,26,53] Fluorescent DNA

dyes can also be used, but this is not the best option because these dyes intercalate into the DNA and require damaging UV light for excitation with unknown effects.[6] Fusion is then obtained using electric pulses or inactivated Sendai viruses.[16] The use of a viral vector and its effects on the embryo and embryo development raises some concerns. Studies demonstrated lack of viral genetic material in the resulting cells, suggesting the absence of potential threats in the offspring or future generations.[53]

Besides the difficulties in the spindle visualisation, its removal is also difficult.[2] The spindle-chromosome complex does not have a membrane, and to prevent chromosome loss during manipulation, a certain volume of ooplasm is also removed.[72] In spite of the inevitability of the co-transfer of some ooplasm, this technique is associated with minimal carryover [6,8,10,26,65] because mitochondria are scattered in the ooplasm and not gathered around the nucleus.[3]

The possibility of using cryopreserved oocytes in this technique, previously only performed with fresh oocytes, has been a major progress.[73] This has allowed to overcome the difficulties related to the lack of synchronism in the retrieval of the oocytes due to differences in the ovarian cycle of the donor and the recipient women and in the responses to gonadotropins and has also allowed to preserve non-used oocytes.[10,73]

Most studies showed that fertilization rates in oocytes obtained using this technique were similar to those of controls.[29,63] Nevertheless, the spindle is very sensitive to micromanipulation,[65,72,73] which frequently induces premature activation of oocytes.[26,39] This can lead to abnormal fertilization due to premature chromatid separation in the absence of the second polar body, resulting in a high incidence of erroneous numbers of pronuclei

formation.[6,10,53,73] It is also crucial to remove the first polar body from the donor oocyte along with the spindle, because it can be reabsorbed during later fusion and cause polyploidy.[53] Despite all this possible complications, aneuploidy rates seem to be similar to controls.[29,52,63]

c. Pronuclear transfer

Pronuclear transfer (PNT) involves fertilization of both the donor and the recipient oocyte. After that, the two pronuclei are removed from each zygote and the ones taken from the parents' embryo are injected into the enucleated donor embryo,[26,28,29,31,71,74] followed by fusion with electric pulses or with inactivated Sendai viruses.[6,16,26,28]

Pronuclei are easier to manage because of the larger volume and the surrounding membrane, but their manipulation may induce greater cellular trauma.[2]

In pronuclear stage, mitochondria are concentrated in the peri-nuclear space, which may lead to higher mutated mtDNA carryover.[3,69] Available data on heteroplasmy are not consistent, with some studies reporting over 20% heteroplasmy,[2,22,72] while others refer less than 2%.[6,8,26,53,65,75] The high percentages of heteroplasmy observed in some studies are mainly justified by mtDNA amplification around pronuclei induced by zygotic activation.[72]

So far, studies have revealed low embryonic development after using this technique, but they have only been performed in abnormally fertilized embryos. Further investigation in normally fertilized embryos is required to clarify whether it is a consequence of the tested embryos or due to the technique.[8,26]

The major disadvantage of this procedure is that both oocytes need to be fertilized and half of the embryos obtained will be discarded during manipulation.[26,28,65,72]

d. Polar body transfer

Polar body transfer (PBT) can be performed either using the first or second polar bodies. While the first polar body can be obtained prior to fertilization, the second polar body requires fertilization to be extracted. These techniques are similar to MST or PNT when using the first polar body or the second polar body, respectively.[65]

Polar bodies theoretically share the same genetic information as the oocyte.[65,72] However, due to the oocyte-biased inheritance of mitochondria, they contain very few cytoplasm and cellular organelles, such as mitochondria.[2,52,65,72] Thus, minimal mutated mtDNA carryover is expected using this technique.[65,72] Indeed, several studies have shown undetectable mutated mtDNA when first PBT is performed, and around 2% when second PBT is used.[2,22,72] Nevertheless, the reduced amount of cytoplasm present in the polar bodies can have deleterious consequences when applying this technique. In first PBT it can result in multipolar spindles and aneuploidy, while in second PBT further research is needed to understand if the spindles' formation is normal, due to the possible absence of centrioles in the residual cytoplasm.[52]

Besides the reduced carryover of mutated mtDNA, another advantage of this technique is the polar body's easy visualization and manipulation without chromosome loss because of its cellular membrane.[2,72] As polar bodies are already separated from the oocyte, manipulation is done without chromosome

damage.[52,65] However, the procedure that uses the second polar body has some difficulties. As the second polar body only contains a haploid genome, removal of the maternal pronucleus of the recipient zygote would be required previously to the transfer. The procedure of removing only one pronucleus is challenging [72] and distinguishing the male and female pronuclei is difficult,[52] so the zygote resulting from fertilization of the donated oocyte should be totally enucleated and then, after the introduction of the second polar body's genome, it should be fertilized again.[72]

There is only one report of first PBT using human oocytes and no reports on second PBT exist.[52] The report on first PBT showed fertilization rates similar to those of controls and potential for onward development [52,72] but blastocyst formation was limited.[52]

If PBT can be successfully performed in parallel with other approaches like MST or PNT, the global efficiency of NT will be increased, reducing by half the number of donor oocytes required.[2,72]

Further studies are still needed to confirm if the incidence of nDNA mutations in polar bodies is identical to that of the sibling oocyte or if their genomes have more defects.[65] Another issue requiring further investigation is the similarity of the epigenetic landscape of these cells.[72]

e. Blastomere transfer

Blastomere transfer consists in the transfer of a blastomere from an affected embryo into an enucleated healthy donor oocyte.[53,69] It is still unclear whether this technique can successfully prevent mtDNA disease in the resulting offspring because an entire cell is fused to the recipient oocyte, instead of only using the nuclear material. This difference may result in higher

levels of heteroplasmy [53] and in addition, embryos may have poor developmental competence.[69]

Besides appearing technically more challenging, this procedure is also associated with more ethical issues than the previously described as it resembles to reproductive cloning. Reproductive cloning can be defined as all procedures that result in the birth of a child genetically identical to an embryo or a born individual. According to this, blastomere transfer could represent reproductive cloning, which does not happen with the previously mentioned NT techniques. A narrower definition has been presented as reproductive cloning only occurring when there is genetic identity with at least one other born individual. If this second definition is considered, blastomere transfer would only be considered reproductive cloning if more than one embryo is transferred and results in pregnancy and birth of at least two children.[69]

Because of all the increased technical and ethical difficulties associated with this technique, it has never been applied clinically and no studies have been reported in humans.[53]

6. Genome editing

Genome editing to prevent mtDNA diseases transmission consists in removing mutated mtDNA by targeting restriction endonucleases in heteroplasmic cells.[76] There are several tools that can be used in this methodology, namely transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 system (CRISPR/Cas-9).[57] These molecules are site specific nucleases that are injected into the cell to selectively target and degrade mtDNA with a specific mutation.[14,30,36,60,77] One of the main advantages of this technique is that donor oocytes are not required.[36,78]

Relying on the principle that less is better, this reduction of mutated mtDNA may constitute and adequate approach.[30] In addition, this technique is less invasive to oocytes than NT.[36] Nevertheless, the amount of mutated mtDNA reduction is variable and the mutation load remaining is still higher than that obtained after NT or PGD [14] and may even be above the threshold for disease manifestation. In order to further reduce the risks of transferring an affected embryo, PGD following this technique is mandatory, preferably using blastocyst biopsy as more cells can be collected.[77]

Besides, the reduction of mtDNA copy number, that can be up to 75% of the starting level,[18] can lead to a mtDNA copy number below a specific threshold necessary for embryonic implantation and development.[18,36]

Side effects may occur when essential genes are cleaved due to off-target editing.[77,78] To avoid this, careful design of the guiding molecules, which are responsible for identifying the sequence targeted, is required.[77]

Further research is still required to establish a proper quantity of nuclease to be injected into the cell in order to guarantee efficiency, while avoiding increasing unnecessary off-target risks.[36,78]

More recently, a new approach was proposed in which, instead of removing the mutated DNA as in the previously mentioned techniques, its sequence is altered. This base editing technique converts one base pair to another at a target locus without requiring double-stranded DNA breaks. It has shown a good efficiency and, when compared to CRISPR/Cas-9, less off-target modifications.[79] This technique has not yet been used in mtDNA, but it may be a potential new method to prevent mitochondrial disorders.

Selection of male embryos

As mitochondrial inheritance is exclusively maternal, selection of male embryos would definitely eliminate transmission for future generations.[14,16,49]

Sex selection for medical reasons, such as preventing diseases with distinct sex incidence or severity, is generally accepted.[80] Sex selection will not reduce the health risks for the second generation, but it will avoid difficult reproductive decisions for them and reduce the risks for the third generation.[81] Allowing this selection is being discussed, as this approach raises not only ethical, but also technical questions due to the requirement of additional manipulation.[3,80]

The selection done pre-fertilization by sperm sorting, selecting only spermatozoa containing an Y chromosome, seems to be safe although not very effective, and so follow up is still required.[3,81] The main advantage of pre-fertilization selection is avoiding to discard female embryos.[81]

The most effective sex selection procedure is PGD, whose major drawback is the destruction of female embryos just based on their sex.[80]

If PGD is used to prevent mtDNA diseases transmission and male selection is considered, different options may be chosen. If one decides to include embryo's sex as the third selection criterion, with the same importance as the mutation load and the embryo quality, 50% of the embryos will be discarded based on their sex, and others will additionally be discarded because of their mutation load or quality. This can make it almost impossible to find a suitable embryo to transfer, leading to the transfer of lower quality embryos or to new treatment cycles. As a consequence, the take-home-baby rate would be negatively affected, and women could end up exposed to greater risks due to the cycles' repetition and the risk of ovarian hyperstimulation syndrome. The alternative option is to include the embryo's sex as a criterion, but not equivalent to the mutation load and the embryo quality. If this is applied, both male and female

embryos with good quality and mutant load below the threshold can be selected, and female embryos will only be transferred when male ones are not available.[81]

If male selection is applied when using NT techniques in which PGD is already being performed to confirm the mutation load, costs will not be heavily increased. However, if PGD is only applied to confirm the embryo's sex, costs will increase and there may be greater risks for the embryo by adding extra manipulation only for this reason. Nevertheless, in both cases, if the decision is to only transfer male embryos and a female one is obtained, the procedure would have to be restarted and more donated oocytes would be necessary.[81]

Because of the technical, economical and ethical implications of this selection, it has been proposed only as an initial approach to these techniques until robust evidence on efficiency and safety is obtained.[3,30] However, this limitation will not allow the study of the effects on future generations.[3] When further evidence is obtained and before the use of female embryos, all these questions will have to be addressed.

Conclusion

As there is still no treatment for diseases caused by mtDNA mutations, prevention is of major importance. Thanks to the development of several techniques of preimplantation diagnosis and intervention, couples can now choose to try to prevent their future children from being affected by these severe diseases. Nevertheless, due to the specificities of the transmission of mtDNA-linked diseases, and owing to the insufficient data on these techniques, it is still questionable whether the information available and transmitted by health professionals to the couple allows them to select the most adequate option. It is also questionable whether couples are able to fully understand the specificities of the transmission of these diseases and potential risks associated with each technique.

In fact, the conditions in which these techniques were tested differ substantially between studies, which makes the comparison between the results difficult. As a consequence, even health professionals still do not have enough data to be certain of which of the techniques will offer the best results in each case. It is necessary to develop further research in which similar conditions are used for all the techniques so that comparable results are obtained in other to improve counselling. Further information is also necessary with regard to side effects and the long-term consequences of these techniques for their safe clinical application.

As ethical issues remain a limitation, boundaries should be defined in order to allow further research in this area, possibly requiring the use of embryos and long-term follow up of children and subsequent generations, but still avoiding the “slippery slope” feared by many.

References

1. Chiaratti MR, Meirelles FV, Wells D, Poulton J. Therapeutic treatments of mtDNA diseases at the earliest stages of human development. Mitochondrion. 2011;11:820–8.

2. Xu L, Shi R. Weigh and wait: the prospect of mitochondrial gene replacement. Hum. Fertil. (Camb). 2016;19:222–9.

3. Appleby JB. The ethical challenges of the clinical introduction of mitochondrial replacement techniques. Med. Health Care. Philos. 2015;18:501–14.

4. Paine A, Jaiswal MK. Promise and Pitfalls of Mitochondrial Replacement for Prevention and Cure of Heritable Neurodegenerative Diseases Caused by Deleterious Mutations in Mitochondrial DNA. Front. Cell. Neurosci. 2016;10:219.

5. Reddy P, Ocampo A, Suzuki K, Luo J, Bacman SR, Williams SL, et al. Selective elimination of mitochondrial mutations in the germline by genome editing. Cell. 2015;161:459–69.

6. Richardson J, Irving L, Hyslop LA, Choudhary M, Murdoch A, Turnbull DM, et al. Concise reviews: Assisted reproductive technologies to prevent transmission of mitochondrial DNA disease. Stem Cells. 2015;33:639–45.

7. Holmes D. New IVF techniques put mitochondrial diseases in focus. Lancet. Neurol. 2014;13:28–9.

8. Tanaka AJ, Sauer M V, Egli D, Kort DH. Harnessing the stem cell potential: the path to prevent mitochondrial disease. Nat. Med. 2013;19:1578–9.

9. Westly E. When powerhouses fail. Nat. Med. 2010;16:625–7.

10. Smeets HJM. Preventing the transmission of mitochondrial DNA disorders: selecting the good guys or kicking out the bad guys. Reprod. Biomed. Online. 2013;27:599–610.

11. Sallevelt SCEH, Dreesen JCFM, Drusedau M, Spierts S, Coonen E, van Tienen FHJ, et al. Preimplantation genetic diagnosis in mitochondrial DNA disorders:

challenge and success. J. Med. Genet. 2013;50:125–32.

12. Koene S, Smeitink J. Mitochondrial medicine: entering the era of treatment. J. Intern. Med. 2009;265:193–209.

13. Hellebrekers DMEI, Wolfe R, Hendrickx ATM, de Coo IFM, de Die CE, Geraedts JPM, et al. PGD and heteroplasmic mitochondrial DNA point mutations: a systematic review estimating the chance of healthy offspring. Hum. Reprod. Update. 2012;18:341–9.

14. Smeets HJM, Sallevelt SCEH, Dreesen JCFM, de Die-Smulders CEM, de Coo IFM. Preventing the transmission of mitochondrial DNA disorders using prenatal or preimplantation genetic diagnosis. Ann. N. Y. Acad. Sci. 2015;1350:29–36.

15. Bianco B, Montagna E. The advances and new technologies for the study of mitochondrial diseases. Einstein (Sao Paulo). 2016;14:291–3.

16. Cree L, Loi P. Mitochondrial replacement: from basic research to assisted reproductive technology portfolio tool-technicalities and possible risks. Mol. Hum. Reprod. 2015;21:3–10.

17. Engelstad K, Sklerov M, Kriger J, Sanford A, Grier J, Ash D, et al. Attitudes toward prevention of mtDNA-related diseases through oocyte mitochondrial replacement therapy. Hum. Reprod. 2016;31:1058–65.

18. Diot A, Dombi E, Lodge T, Liao C, Morten K, Carver J, et al. Modulating mitochondrial quality in disease transmission: towards enabling mitochondrial DNA disease carriers to have healthy children. Biochem. Soc. Trans. 2016;44:1091–100.

19. Poulton J, Chiaratti MR, Meirelles F V, Kennedy S, Wells D, Holt IJ. Transmission of mitochondrial DNA diseases and ways to prevent them. PLoS Genet. 2010;6.

20. Poulton J, Kennedy S, Oakeshott P, Wells D. Preventing transmission of maternally inherited mitochondrial DNA diseases. BMJ. 2009;338:b94.

21. Samuels DC, Wonnapinij P, Chinnery PF. Preventing the transmission of pathogenic mitochondrial DNA mutations: Can we achieve long-term benefits from germ-line gene transfer? Hum. Reprod. 2013;28:554–9.

22. Gemmell N, Wolff JN. Mitochondrial replacement therapy: Cautiously replace the master manipulator. Bioessays. 2015;37:584–5.

23. Bredenoord AL, Pennings G, de Wert G. Ooplasmic and nuclear transfer to prevent mitochondrial DNA disorders: conceptual and normative issues. Hum. Reprod. Update. 2008;14:669–78.

24. St John JC, Campbell KHS. The battle to prevent the transmission of mitochondrial DNA disease: is karyoplast transfer the answer? Gene Ther. 2010. p. 147–9.

25. Mitalipov S, Amato P, Parry S, Falk MJ. Limitations of preimplantation genetic diagnosis for mitochondrial DNA diseases. Cell Rep. 2014. p. 935–7.

26. Amato P, Tachibana M, Sparman M, Mitalipov S. Three-parent in vitro fertilization: gene replacement for the prevention of inherited mitochondrial diseases. Fertil. Steril. 2014;101:31–5.

27. Bredenoord AL, Dondorp W, Pennings G, De Die-Smulders CEM, De Wert G. PGD to reduce reproductive risk: the case of mitochondrial DNA disorders. Hum. Reprod. 2008;23:2392–401.

28. Yabuuchi A, Beyhan Z, Kagawa N, Mori C, Ezoe K, Kato K, et al. Prevention of mitochondrial disease inheritance by assisted reproductive technologies: prospects and challenges. Biochim. Biophys. Acta. 2012;1820:637–42.

29. Mitalipov S, Wolf DP. Clinical and ethical implications of mitochondrial gene transfer. Trends Endocrinol. Metab. 2014;25:5–7.

30. Falk MJ, Decherney A, Kahn JP. Mitochondrial Replacement Techniques--Implications for the Clinical Community. N. Engl. J. Med. 2016;374:1103–6.

31. Liu H-S, Chu P-L. Three-parent embryo: The therapeutic future for inherited mitochondrial diseases. J. Formos. Med. Assoc. 2015;114:1027–8.

32. Sallevelt SCEH, Dreesen JCFM, Drusedau M, Hellebrekers DMEI, Paulussen ADC, Coonen E, et al. PGD for the m.14487 T>C mitochondrial DNA mutation resulted in the birth of a healthy boy. Hum. Reprod. 2017;32:698–703.

33. Dimond R. Social and ethical issues in mitochondrial donation. Br. Med. Bull. 2015;115:173–82.

34. Varvastian S. UK's Legalisation of Mitochondrial Donation in IVF Treatment: A Challenge to the International Community or a Promotion of Life-saving Medical Innovation to Be Followed by Others? Eur. J. Health Law. 2015;22:405–25.

35. Sallevelt SCEH, Dreesen JCFM, Coonen E, Paulussen ADC, Hellebrekers DMEI, de Die-Smulders CEM, et al. Preimplantation genetic diagnosis for mitochondrial DNA mutations: analysis of one blastomere suffices. J. Med. Genet. 2017;54:693–7.

36. Wang S, Yi F, Qu J. Eliminate mitochondrial diseases by gene editing in germ-line cells and embryos. Protein Cell. 2015;6:472–5.

37. Santos TA, El Shourbagy S, St. John JC. Mitochondrial content reflects oocyte variability and fertilization outcome. Fertil. Steril. 2006;85:584–91.

38. Poulton J, Bredenoord AL. 174th ENMC international workshop: Applying pre-implantation genetic diagnosis to mtDNA diseases: implications of scientific advances 19-21 March 2010, Naarden, The Netherlands. Neuromuscul. Disord. 2010. p. 559–63.

39. Hyslop LA, Blakeley P, Craven L, Richardson J, Fogarty NME, Fragouli E, et al. Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease. Nature. 2016;534:383–6.

40. Hens K, Dondorp W, de Wert G. A leap of faith? An interview study with professionals on the use of mitochondrial replacement to avoid transfer of mitochondrial diseases. Hum. Reprod. 2015;30:1256–62.

41. Reinhardt K, Dowling DK, Morrow EH. Medicine. Mitochondrial replacement, evolution, and the clinic. Science. 2013;341:1345–6.

42. Vogel G. Assisted reproduction. FDA considers trials of “three-parent embryos”. Science. 2014. p. 827–8.

43. Burgstaller JP, Johnston IG, Poulton J. Mitochondrial DNA disease and developmental implications for reproductive strategies. Mol. Hum. Reprod. 2015;21:11–22.

44. Bredenoord A, Dondorp W, Pennings G, de Die-Smulders C, Smeets B, de Wert G. Preimplantation genetic diagnosis for mitochondrial DNA disorders: ethical guidance for clinical practice. Eur. J. Hum. Genet. 2009;17:1550–9.

45. Heindryckx B, Neupane J, Vandewoestyne M, Christodoulou C, Jackers Y, Gerris J, et al. Mutation-free baby born from a mitochondrial encephalopathy, lactic acidosis and stroke-like syndrome carrier after blastocyst trophectoderm preimplantation genetic diagnosis. Mitochondrion. 2014;18:12–7.

46. De Rycke M. Singling out genetic disorders and disease. Genome Med. 2010;2:74.

47. Bredenoord AL, Pennings G, Smeets HJ, de Wert G. Dealing with uncertainties: ethics of prenatal diagnosis and preimplantation genetic diagnosis to prevent mitochondrial disorders. Hum. Reprod. Update. 2008;14:83–94.

48. Gigarel N, Hesters L, Samuels DC, Monnot S, Burlet P, Kerbrat V, et al. Poor correlations in the levels of pathogenic mitochondrial DNA mutations in polar bodies versus oocytes and blastomeres in humans. Am. J. Hum. Genet. 2011;88:494–8.

49. Treff NR, Campos J, Tao X, Levy B, Ferry KM, Scott RTJ. Blastocyst preimplantation genetic diagnosis (PGD) of a mitochondrial DNA disorder. Fertil. Steril. 2012;98:1236–40.

50. Claiborne AB, English RA, Kahn JP. ETHICS OF NEW TECHNOLOGIES. Finding an ethical path forward for mitochondrial replacement. Science.

2016;351:668–70.

51. Bredenoord AL, Krumeich A, De Vries MC, Dondorp W, De Wert G. Reproductive decision-making in the context of mitochondrial DNA disorders: views and experiences of professionals. Clin. Genet. 2010;77:10–7.

52. Craven L, Tang M-X, Gorman GS, De Sutter P, Heindryckx B. Novel reproductive technologies to prevent mitochondrial disease. Hum. Reprod. Update. 2017;23:501–19.

53. Reznichenko AS, Huyser C, Pepper MS. Mitochondrial transfer: Implications for assisted reproductive technologies. Appl. Transl. genomics. 2016;11:40–7.

54. Wise J. Mitochondrial donation is "not unsafe," review confirms. BMJ. 2014. p. g3723.

55. Murray TH. Genetics. Stirring the simmering "designer baby" pot. Science. 2014;343:1208–10.

56. Tavare A. Scientists are to investigate "three parent IVF" for preventing mitochondrial diseases. BMJ. 2012. p. e540.

57. Zhang X, Wang S. From the first human gene-editing to the birth of three-parent baby. Sci. China. Life Sci. 2016;59:1341–2.

58. Hawkes N. UK clinics may be able to offer mitochrondrial donation next spring. BMJ. 2016. p. i6492.

59. Torjesen I. Government gives the go ahead for mitochondrial donation during IVF. BMJ. 2014. p. g4801.

60. Klopstock T, Klopstock B, Prokisch H. Mitochondrial replacement approaches: challenges for clinical implementation. Genome Med. 2016;8:126.

61. Cohen IG, Adashi EY. Preventing Mitochondrial DNA Diseases: One Step Forward, Two Steps Back. JAMA. 2016;316:273–4.

62. Callaway E. Three-person embryos may fail to vanquish mutant mitochondria.

Nature. 2016. p. 445–6.

63. Kang E, Wu J, Gutierrez NM, Koski A, Tippner-Hedges R, Agaronyan K, et al. Mitochondrial replacement in human oocytes carrying pathogenic mitochondrial DNA mutations. Nature. 2016;540:270–5.

64. Herbert M, Turnbull D. Mitochondrial Donation - Clearing the Final Regulatory Hurdle in the United Kingdom. N. Engl. J. Med. 2017;376:171–3.

65. Wei Y, Zhang T, Wang Y-P, Schatten H, Sun Q-Y. Polar bodies in assisted reproductive technology: current progress and future perspectives. Biol. Reprod. 2015;92:19.

66. Darnovsky M. A slippery slope to human germline modification. Nature. 2013;499:127.

67. Shoubridge EA. Biomedicine: Replacing the cell's power plants. Nature. 2016;540:210–1.

68. Zhang J, Liu H, Luo S, Lu Z, Chavez-Badiola A, Liu Z, et al. Live birth derived from oocyte spindle transfer to prevent mitochondrial disease. Reprod. Biomed. Online. 2017;34:361–8.

69. Bredenoord AL, Dondorp W, Pennings G, De Wert G. Nuclear transfer to prevent mitochondrial DNA disorders: revisiting the debate on reproductive cloning. Reprod. Biomed. Online. 2011;22:200–7.

70. Otsuki J, Nagai Y, Sankai T. Aggregated chromosomes transfer in human oocytes. Reprod. Biomed. Online. 2014;28:401–4.

71. Pitts-Tucker T. UK fertilisation authority launches consultation on mitochondrial replacement techniques. BMJ. 2012. p. e6259.

72. Wang T, Sha H, Ji D, Zhang HL, Chen D, Cao Y, et al. Polar body genome transfer for preventing the transmission of inherited mitochondrial diseases. Cell. 2014;157:1591–604.

73. Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, et al. Towards germline gene therapy of inherited mitochondrial diseases. Nature. 2013;493:627–31.

74. Hammond ER, Green MP, Shelling AN, Berg MC, Peek JC, Cree LM. Oocyte mitochondrial deletions and heteroplasmy in a bovine model of ageing and ovarian stimulation. Mol. Hum. Reprod. 2016;22:261–71.

75. Shukla J. Technique to eliminate mitochondrial disease carries very small risk of failure, researchers find. BMJ. 2016. p. i3288.

76. Kyriakouli DS, Boesch P, Taylor RW, Lightowlers RN. Progress and prospects: gene therapy for mitochondrial DNA disease. Gene Ther. 2008;15:1017–23.

77. Ishii T. Reproductive medicine involving genome editing: clinical uncertainties and embryological needs. Reprod. Biomed. Online. 2017;34:27–31.

78. Pang RTK, Ho PC. Designer babies. Obstet. Gynaecol. Reprod. Med. 2016;26:59–60.

79. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature [Internet]. Nature Publishing Group; 2017;1–27. Available from: http://www.nature.com/doifinder/10.1038/nature24644

80. Leiter G. What Israeli policy can teach us about elective sex selection. Isr. J. Health Policy Res. 2014;3:42.

81. Bredenoord AL, Dondorp W, Pennings G, De Wert G. Avoiding transgenerational risks of mitochondrial DNA disorders: a morally acceptable reason for sex selection? Hum. Reprod. 2010;25:1354–60.