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Review

The role of mitochondrial activity in female fertility and assisted reproductive technologies: overview and current insights

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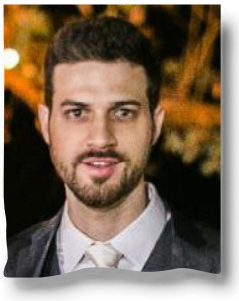

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KEY MESSAGE

Reproductive medicine experts must be aware of the role of mitochondria on female fertility and ART, not only to ensure a better clinical practice, but also to provide updated information upon a patient's request. Imminent therapeutic alternatives will probably evoke a new phase for infertility treatment.

A B S T R A C T

Mitochondria have been implicated as key factors regulating female reproductive processes. Notable progress has been made in determining the role of mitochondria with respect to oocyte maturation, fertilization and early embryo development. In addition, mitochondrial function and dysfunction has been the subject of various studies in ovarian ageing and metabolic stress models. However, the overall mitochondrial impact on female fertility is yet to be uncovered. The mitochondrial DNA content of granulosa, cumulus and trophectoderm cells is being explored as a biomarker of oocyte quality and embryo viability. As growing evidence suggests that embryo potential could be related to the ability of oocyte mitochondria to generate energy, efforts have been made to investigate the possibility of improving mitochondrial capacity in women with poor outcomes after treatment with assisted

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reproductive technologies. Thus far, therapeutic attempts have focused mainly on using nutrients to restore mitochondrial function and transferring mitochondria from autologous germline precursor cells. Moreover, new perspectives on optimizing infertility treatments have arisen with modern mitochondrial replacement therapies, which are being applied in women with mitochondrial disease-causing mutations. This review explores aspects of the distinctive contribution of mitochondria to reproductive processes and discusses current and emerging clinical implications.

Introduction

The mitochondrion is a small organelle exclusively of maternal inheritance and is directly involved in many essential cellular functions, including energy synthesis, management of reactive oxygen species (ROS) and apoptotic regulatory control. The mature human oocyte contains more mitochondria and mitochondrial DNA (mtDNA) than other cell types, and several hypotheses implicate mitochondria as key factors mediating reproductive competence (Benkhalifa et al., 2014; Ramalho-Santos et al., 2009; Schatten et al., 2014).

Female fertility is one of the first body functions to be affected by ageing. Reproductive capacity peaks at approximately 25 years of age and declines slightly by the age of 32, the decline accelerating after the age of 37 (Bentov and Casper, 2013; O'Connor et al., 1998). Clinical and experimental data, including the success of egg donation programmes, suggest that decreased oocyte quality is the main factor culminating in the age-related deterioration of reproductive capacity. Although the molecular mechanisms underlying this decrease in egg quality remain poorly understood, altered mitochondrial function has been implicated (Bentov et al., 2011; Wilding et al., 2005). In addition, despite notable research on mitochondrial function with respect to oocyte maturation, fertilization and early embryo development, the overall mitochondrial impact on human infertility remains unclear.

This review discusses from an energy-based perspective how mitochondria affect female reproductive biology and the clinical implications of this theme regarding assisted reproductive technologies (ART), along with emerging mitochondrial therapeutic options. A strategic literature search was performed in PubMed to access the MEDLINE database, for original articles and reviews using the following combined descriptors: 'mitochondria', 'oocytes', 'reproductive techniques, assisted' and 'fertility'. Additional references were collected from the most relevant articles of interest.

Parental mitochondrial inheritance

Mitochondrial genome inheritance does not reflect a Mendelian pattern. Although transmitted solely from the mother, rare exceptions may occur (Cummins, 2000; Schwartz and Vissing, 2002; Song et al., 2014). Studies in animal models and humans have demonstrated that, after fertilization, paternal mitochondria are not detected in embryos beyond the 4-cell stage (Cummins, 2000; Sutovsky et al., 2000). Thus far, no evolutionary theory has been able to present concrete evidence to explain this selective inheritance pattern, and the exact succession of events responsible for extinguishing paternal mitochondria is not fully understood; multiple mechanisms are possibly linked. The most reasonable hypothesis comprises the coexistence of a specific ubiquitin-based proteolytic mechanism (Sutovsky et al., 1999) and fertilization-triggered ooplasmic lysosomal mitophagy (Sato and Sato, 2011).

During spermatogenesis in several mammalian species, the mitochondria of the secondary spermatocyte express ubiquitin, a universal and reliable marker of proteolysis. However, ubiquitin detection is precluded by the natural exposure of sperm to substances that induce the formation of a disulphide bond as the spermatocyte passes through the epididymis. As fertilization occurs, ubiquitin is once again detectable due to disulphide bond reducing agents, especially glutathione. Sperm mitochondria tagging by polyubiquitination allows its recognition and active degradation by the 26S proteasome (Sutovsky et al., 2000).

In 2011, Sato and Sato identified a fertilization-triggered mechanism for paternal mitochondria eradication in animals. Through successive experiments in *Caenorhabditis elegans*, a nematode species, they demonstrated the induction of autophagosome formation immediately after sperm entry. This was followed by the envelopment of sperm-derived mitochondria and targeted lysosomal degradation in the early embryo (Sato and Sato, 2011). The use of a lysosome inhibitor allows for paternal mitochondria persistence until late embryonic stages (Zhou et al., 2011). Furthermore, the mutation or depletion of autophagy regulators is associated with abnormal embryogenesis and diminished embryo survival (Sato and Sato, 2011).

Synchronal activity of such molecular mechanisms has been acknowledged not only for the elimination of paternal mtDNA, but also for the removal of damaged mitochondria (Song et al., 2014, 2016). The first description of an inner mitochondrial membrane mitophagy receptor, prohibitin 2, recently contributed to a new and better understanding of paternal mitochondria clearance (Wei et al., 2017). The role of well-known apoptotic endonucleases is also being clarified (Zhou et al., 2016).

Mitochondrial function in the oocyte and early embryo

Energy production: the need for ATP

Human living cells must produce adequate amounts of the preferred available energy source adenosine triphosphate (ATP). Although there are a number of metabolic pathways for ATP production, 88% of ATP generated from glucose is provided by mitochondrial oxidative phosphorylation (OXPHOS), with the remainder coming from glycolysis and the tricarboxylic acid cycle (Seyfried and Shelton, 2010). Severe deficiency of cellular ATP content often leads to cell apoptosis (Vander Heiden et al., 2009).

All of the complex processes the oocyte goes through prior to ovulation and fertilization require energy, which is derived mainly from ATP production via OXPHOS (Ben-Meir et al., 2015). Early embryo development and implantation potential have been correlated with mitochondrial function and activity (Benkhalifa et al., 2014; Pang et al., 2013). Furthermore, higher ATP content from human oocytes and embryos has been correlated with better reproductive results among infertile patients (Zhao and Li, 2012).

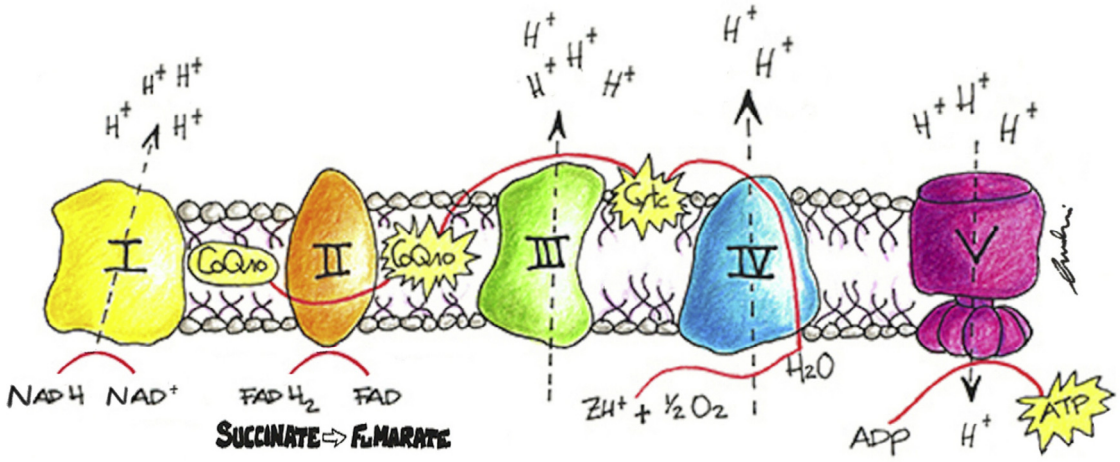

Figure 1 – Electron transport chain (ETC) in the inner mitochondrial membrane. Reactions in complexes I, III and IV result in a stream of protons into the mitochondrial intermembrane space. In complex V, the energy generated by the mobilization of protons back into the mitochondrial matrix results in ATP synthesis by phosphorylation. NADH: nicotinamide adenine dinucleotide hydride, NAD^+: nicotinamide adenine dinucleotide, CoQ10: coenzyme Q10, $FADH_2$: flavin adenine dinucleotide hydride, FAD: flavin adenine dinucleotide, Cytc: cytochrome c, ADP: adenosine diphosphate; ATP: adenosine triphosphate.

The mitochondria of maturing oocytes and pre-implantation embryos undergo qualitative structural transformations and changes in cytoplasmic distribution. In oocytes, the mitochondria tend to be oval and have a dense matrix with few cristae. Central clumping around the pronuclei is also observed (Motta et al., 2003; Sathananthan, 1994; Sathananthan and Trounson, 2000). Beyond the 8-cell stage, a differentiation into swollen organelles with elongated ridges is accompanied by a decrease in the matrix density and an increase in both the number of cristae and the membrane potential. Similarly, they are peripherally distributed to be apart from the mitotic and meiotic spindles (Sathananthan and Trounson, 2000). These structural changes result in a highly active oxidative metabolism and ATP production, impacting oocyte quality and embryo development (Ge et al., 2012). This concept is in agreement with Van Blerkom's observation that mitochondria from some human embryos that fail to develop remain morphologically unchanged (Van Blerkom, 1989).

Oocyte energy production relies essentially on pyruvate as a substrate, leading to ATP generation through oxidative phosphorylation within the electron transport chain (ETC). Glycolysis in oocytes is limited by low phosphofructokinase expression (Collado-Fernandez et al., 2012; Dalton et al., 2014; Wilding et al., 2002). In contrast, cumulus cells have good glycolytic capacity; they confer metabolic support to oocytes by providing pyruvate via gap junctions (Collado-Fernandez et al., 2012; Dalton et al., 2014; Downs, 1995). Pyruvate is the primary substrate that derives from the cumulus cells to the oocyte to generate ATP. Other amino acids and intermediate metabolites are potentially involved (Collado-Fernandez et al., 2012; Xie et al., 2016).

The ETC resides in the inner mitochondrial membrane and comprises five large complexes (**Figure 1**). In complex I, the nicotinamide adenine dinucleotide hydride (NADH) generated in the Krebs cycle undergoes oxidation. An induced reduction of flavin adenine dinucleotide hydride ($FADH_2$) to flavin adenine dinucleotide (FAD) by the electrons released during the conversion of succinate to fumarate occurs in complex II. Ubiquinone, known as coenzyme Q10 (CoQ10), becomes active after being modified by the electrons released by both of the previously described complexes. Subsequently, complex III transfers electrons from the already reduced CoQ10 to cytochrome c, which transfers them to complex IV, where they react with oxygen to form water. The free energy dissipated throughout this process results in a stream of protons into the mitochondrial intermembrane space. Finally, the energy generated by the mobilization of protons back into the mitochondrial matrix results in ATP synthesis by phosphorylation of adenosine diphosphate in complex V (Balaban et al., 2005; Gautheron, 1984).

Surrogate markers of oocyte competence and favourable reproductive outcomes in ART are being sought, especially in transcriptome, proteome and metabolome studies. The analysis of granulosa and cumulus cells is considered one of the best non-invasive strategies available today (Boucret et al., 2015). These cells create an appropriate microenvironment for follicular maturation, fertilization and embryo cleavage (Dumesic et al., 2015; Tsai et al., 2010). Moreover, the signalling routes between cumulus cells and oocytes are bidirectional. Results obtained thus far demonstrate that oocyte viability is most likely correlated with the proper balance of these multiple pathways (Huang and Wells, 2010).

Numerous studies have shown a strong association between the proper functioning of cumulus cells and oocyte quality. Recent publications suggest that the mitochondrial function of cumulus cells can directly influence the ability to achieve a successful pregnancy (Dalton et al., 2014; Huang and Wells, 2010; Tsai et al., 2010). In 2014, Hsu et al. (2014) demonstrated for the first time that women with surgically confirmed endometriosis who undergo ART have reduced ATP production by cumulus cells. In the same group of patients, a positive correlation was found between these findings and the number of mature oocytes, as well as implantation rates. The authors speculated that the results implicated mitochondrial dysfunction.

Ovarian stimulation protocols are universally used in different ART. Yet the hormones utilized in ovarian stimulation may not be completely innocuous to the female reproductive system (Baker et al., 2015; Chappel, 2013). Requena et al. (2016) found that the rate of cumulus cell apoptosis may vary according to the type of gonadotrophin used for ovarian stimulation. Therefore, different stimulation protocols could have a distinct impact on mitochondrial energy production and,

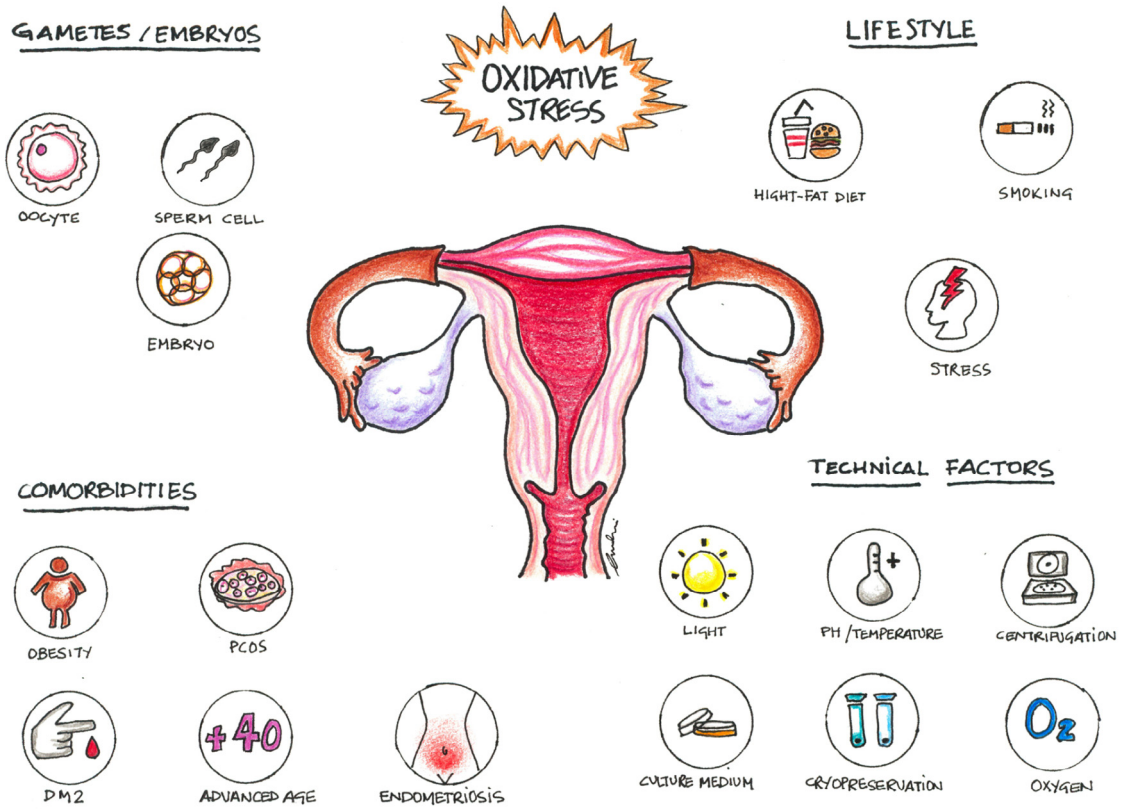

Figure 2 – Oxidative stress-related factors and pathologies with a negative impact on female reproductive processes and assisted reproductive technology (ART) outcomes. Technical issues are well documented with the laboratory manipulation of gametes and embryos. There is increasing evidence that lifestyle habits and specific pathologies that generate oxidative damage also affect the female reproductive system and, ultimately, the success of ART.

ultimately, oocyte quality (Dell'Aquila et al., 2009; Eichenlaub-Ritter et al., 2011).

Various attempts have been made to evaluate mitochondrial function in human embryos. Through the determination of the oxygen consumption rate (OCR), investigators indirectly assessed the activity of mitochondrial OXPHOS; the respiratory function is enhanced as embryos develop, peaking at the blastocyst stage (Hashimoto et al., 2017; Kurosawa et al., 2016). In addition, the OCR of the embryos that progressed to blastocysts was significantly higher than the OCR of embryos in developmental arrest (Kurosawa et al., 2016). It is also relevant that the blastocyst's inner cell mass produces less ATP (Houghton, 2006) and consumes less oxygen (Hashimoto et al., 2017; Houghton, 2006) than trophectoderm cells, exhibiting a quieter metabolism in accordance with Leese's theory (Leese, 2002).

Effects of oxidative stress

Oxidative stress (OS) comes from a condition that alters the proper balance between ROS production and neutralizing antioxidant molecules (Agarwal et al., 2006). Enhanced and unbalanced ROS production may be a predominant cause of impaired mitochondrial OXPHOS (Lord and Aitken, 2013). External factors contribute to higher OS in ART, including exposure to visible light, non-ideal pH and temperature, centrifugation, cryopreservation, culture media composition, oxygen concentrations, and gamete and embryo manipulation (Agarwal et al., 2014). Physical and mental stress related to lifestyle behaviours have also been linked to OS and poorer reproductive outcomes (Prasad et al., 2016). The factors related to OS with an impact on ART outcomes are shown in **Figure 2**.

Tarín first postulated that age-related ROS accumulation and subsequent oxidative damage can affect oocyte fertilization and embryo development (Tarín, 1996). Several elements of OS make mtDNA particularly susceptible, which could lead to critical loss of function and, ultimately, to a diminished capacity to generate ATP (Lord and Aitken, 2013; Zhang et al., 2006). Mammalian mature oocytes and zygotes subjected to mild to intense OS have demonstrated significant mitochondrial dysfunction and ultrastructural changes. A clear impact on both mitochondrial ATP synthesis and the activation of its apoptotic mechanisms has been observed (Liu et al., 2000; Zhang et al., 2006). Conversely, granulosa cells and follicular fluid provide a favourable microenvironment with distinct antioxidants (Eichenlaub-Ritter et al., 2011). Despite increased levels of antioxidant enzymes within ovarian tissue, mice exposed to long-term OS present with not only a substantially higher percentage of defective mitochondria in follicular granulosa cells, but also lower fertility and fecundity rates (Shi et al., 2016).

Babayev et al. (2016) reported increased expression of mitochondrial stress-related genes in older mouse oocytes, along with accumulated ROS content and insufficient antioxidant capacity. Similarly, in patients over 38 years of age, most of the luteal granulosa cells from periovulatory follicles exhibit an abnormal mitochondrial morphology and reduced expression of crucial antioxidant enzymes compared with women younger than 32 years of age (Tatone et al., 2006).

Traditionally, aneuploidy of embryos is considered to be the main factor associated with lower implantation potential and higher rates of miscarriage in women at advanced reproductive age. The pathogenesis of human aneuploidy is thought to be multifactorial and may be related to defective steps during meiosis I or II. Perkins et al. (2016) described the sequelae of inhibiting superoxide dismutase in the female germline. The lack of such a potent antioxidant induces errors in the segregation of homologous chromosomes due to the loss of sister chromatid cohesion. Because maternal ageing aggravates oxidative damage, these data offer a plausible explanation for the origin of aneuploidy gametes in older women. Consistently, the frequency of chromosomal segregation errors during meiosis I in human oocytes increases with age (Nagaoka et al., 2012).

In addition, as shown by Zhang et al. (2006), lower mitochondrial ATP synthesis may cause aberrations on the meiotic spindles of mature oocytes and result in disrupted chromosome alignment. Accordingly, a higher incidence of errors in the second meiotic division has been shown to be at least partially related to mitochondrial dysfunction (Zeng et al., 2007; Zhang et al., 2006).

Biogenesis of mtDNA and copy number

Low ATP levels in oocytes are associated with reduced embryo viability and implantation potential (Quinn and Wales, 2006; Van Blerkom et al., 1995). Therefore, embryo viability has been suggested to be related directly to oocyte mitochondrial activity and the ability to produce energy (May-Panloup et al., 2005; Reynier et al., 2001; Zhao and Li, 2012). Within this context, mtDNA copy number has been proposed as a surrogate measure of mitochondrial function.

Oocyte mtDNA increases until the stage that immediately precedes fertilization, which coincides with ovulation. In healthy embryos, the accumulated mtDNA is divided equally among all cells during embryogenesis. Thus, the mtDNA copy number per cell progressively diminishes, whereas the cellular requirements for ATP apparently increase. This may be particularly relevant, as the replication of mtDNA will only resume at the blastocyst stage (Chappel, 2013; Fragouli et al., 2015; St. John et al., 2010; Wai et al., 2010).

During initial embryo cleavage, the mitochondrial distribution between blastomeres may be unequal. Whenever it occurs, cells receiving less mtDNA exhibit a reduced bioenergetic capacity and consequently evolve to lysis and death (Van Blerkom, 2011). These findings have been reported not only in animal blastocysts (Katayama et al., 2006; Squirrell et al., 2003); experimentally, an asymmetric distribution pattern affecting the developmental competence has also been shown in human embryos (Van Blerkom et al., 2000).

Recent studies have proposed quantifying mtDNA in cumulus, granulosa and trophectoderm cells to predict embryo quality and viability (Desquiret-Dumas et al., 2017; Diez-Juan et al., 2015; Fragouli et al., 2015; Ogino et al., 2016). Boucret et al. (2015) concluded that the mtDNA content in cumulus cells is correlated with the amount detected within the oocyte for each cumulus–oocyte complex. Consequently, they suggested that the mitochondrial characteristics of such cells can serve as markers of oocyte competence. Dumesic et al. (2016) showed a positive correlation between the ability of the mitochondrial membrane potential of cumulus cells to resist stress and the number of collected mature oocytes. Notably, these studies had some limitations; apart from performing a stimulated cycle, the analysis involved either immature oocytes or pooled cumulus cells (Boucret et al., 2015; Dumesic et al., 2016). An association between higher mtDNA amounts in cumulus cells and good-quality embryos has been reported (Desquiret-Dumas et al., 2017; Ogino et al., 2016).

Furthermore, mtDNA mutations or deletions have been correlated with organelle dysfunction, low ATP levels and embryonic developmental arrest (Tsai et al., 2010). As women age, more mtDNA deletions in luteinized granulosa and cumulus cells are accompanied by lower pregnancy rates (Seifer et al., 2002; Tsai et al., 2010). Taken together, these findings corroborate the understanding that granulosa and cumulus cell mitochondrial status directly influences embryonic development, as well as the capacity for the activation, maturation and fertilization of oocytes.

Fertilized oocytes have a considerably greater amount of mtDNA (Reynier et al., 2001; Santos et al., 2006; Zeng et al., 2007), whereas oocytes from older women or from those who present with ovarian insufficiency express fewer mtDNA copies prior to fertilization (Duran et al., 2011; Fragouli et al., 2015; May-Panloup et al., 2005). In contrast, mtDNA copy number at the blastocyst stage for both euploid and aneuploid embryos is higher in older women (Fragouli et al., 2015). Importantly, aneuploid blastocysts have a higher mitochondrial copy number than euploid blastocysts. The same has been observed with euploid blastocysts that fail to implant compared with those that implant (Diez-Juan et al., 2015; Fragouli et al., 2015).

A blinded retrospective assessment of mtDNA levels in blastocyst biopsies from 282 euploid embryos found an implantation rate of 74.3% among those with low or normal mtDNA levels. In contrast, all 33 embryos presenting with elevated levels of mtDNA failed to implant, revealing a 100% negative predictive value. The authors assert that appropriate and validated mtDNA quantification could be used as a biomarker of viability in euploid embryos (Ravichandran et al., 2017). Later, a blinded prospective non-selection study from the same group provided evidence supporting this concept (Fragouli et al., 2017).

Notably, these studies were recently challenged by Victor et al. (2017), who found no significant difference between blastocyst mtDNA scores when compared for embryo viability, ploidy or maternal age. Also, the only study controlling for patient-specific variables failed to demonstrate any predictive value of mtDNA content with respect to reproductive potential. In this retrospective analysis, no difference was detected when trophectoderm mtDNA levels were compared between euploid embryos which were transferred together but resulted in a singleton (Treff et al., 2017).

Mitochondrial hyperproliferation is likely to be indicative of a compensatory mechanism for intracellular metabolic stress. Embryos that are subject to energy depletion may increase mtDNA biogenesis in an attempt to compensate for energy deficiency (Diez-Juan et al., 2015; Fragouli et al., 2015; May-Panloup et al., 2016). However, such aberrant and unregulated replication does not necessarily reflect better ATP production because these mitochondria are most likely dysfunctional (Fragouli et al., 2015; Grindler and Moley, 2013). Whether mtDNA copy number in granulosa, cumulus or trophectoderm cells is a reliable and helpful test in assisted reproduction will need to be studied further using randomized prospective trial designs.

Pathologies that affect female fertility through metabolic stress

Senescence stands out among the predisposing factors for embryonic metabolic stress. Studies in animals have shown that, in addition to having morphological and ultrastructural modifications, mitochondria from older animal oocytes generate more ROS and less ATP. In order to explain the age-related changes in mitochondrial function,

different mechanisms that lead to the disturbance of mitochondrial homeostasis have been interrogated. Aside from mtDNA mutations and deletions (Duran et al., 2011; El Shourbagy et al., 2006; Simsek-Duran et al., 2013), other aspects of mitochondrial function, such as a dysfunctional ETC or unbalanced mitochondrial calcium concentration, could be involved (Ziegler et al., 2015).

The detrimental impact of obesity on ART is becoming an area of extensive scientific investigation. Differences in endometrial gene expression, oocyte and embryo quality, and the number of retrieved oocytes have been documented in obese women compared with those who are normal weight, leading to lower fertilization, implantation and clinical pregnancy rates (Bellver et al., 2010; Broughton and Moley, 2017; Comstock et al., 2017; Gu et al., 2015; Luke et al., 2011; Maheshwari et al., 2007; Purcell and Moley, 2011).

Metabolic stress affects mitochondrial function, and its related consequences are extensively described herein. Similar mechanisms have been reported in animal and human studies in relation to not only ageing (Babayev et al., 2016; Duran et al., 2011) and obesity (Grindler and Moley, 2013; Purcell and Moley, 2011; Wu et al., 2015), but also insulin resistance (Ou et al., 2012; Purcell and Moley, 2011), diabetes (Wang et al., 2009, 2010), psychological stress (Prasad et al., 2016), endometriosis (Prieto et al., 2012), the classic form of polycystic ovary syndrome (PCOS) (Harris et al., 2010), high-fat diets (Luzzo et al., 2012; Wu et al., 2010) and smoking (Paszkowski et al., 2002) (**Figure 2**).

Emerging therapeutic alternatives

Bioactive molecules, antioxidants and calorie restriction

Recently, efforts have been made to investigate the possibility of improving mitochondrial function by using bioactive molecules, which protect against oxidative damage and increase the overall efficiency of energy production. The main bioenergetic mitochondrial nutrients are summarized in **Table 1**.

Calorie restriction has also been proposed as a conceivable strategy to ameliorate female fertility capacity in selected cases (Igarashi et al., 2015; Tilly and Sinclair, 2013). Experiments in rodents have shown that, when their usual calorie intake is restricted by 40%, the occurrence of germline cell division errors and mitochondrial damage significantly decreases (Selesniemi et al., 2011). Similarly, a retrospective cohort study including 120 patients showed that a diet composed of a protein intake greater than 25% combined with a carbohydrate intake lower than 40% is related to better blastocyst development and pregnancy rates (Russell et al., 2012). However, a recent systematic review concluded that there is not enough evidence to support low-carbohydrate diets for infertile women without PCOS (McGrice and Porter, 2017).

In the clinical setting, the latest Cochrane review on oral antioxidant supplementation did not find any effect on live birth or clinical pregnancy rates (Showell et al., 2013), and a systematic review and meta-analysis on the use of melatonin proved imprecise to determine its real impact on ART (Seko et al., 2014). Properly designed randomized clinical trials that are forthcoming should shed some light on this area and provide additional information.

Overall, the available evidence does not allow a categorical recommendation for the use of in-vivo nutrient supplies to improve reproductive outcomes. Egg donation remains the primary therapy for cases of multiple failures caused by a female infertility factor.

Table 1 – The most widely studied bioactive nutrients for optimizing mitochondrial function.

	Strategy	Possible effects on fertility
Abdulhasan *et al.*, 2017; Ben-Meir *et al.*, 2015; Bentov *et al.*, 2014; Boots *et al.*, 2016	Coenzyme Q10	Lowers the aneuploidy rate[a] Delays ovarian reserve depletion Restores oocyte mitochondrial gene expression Improves mitochondrial activity and distribution Lowers the ROS level in oocytes Increases mitochondrial mass and polarization Increases ATP levels in oocytes
Liu *et al.*, 2013; Sugiyama *et al.*, 2015; Takeo *et al.*, 2014	Resveratrol	Increases the follicle pool and embryo development Improves the number and quality of oocytes Improves fertilization and developmental ability Increases ATP in oocytes Increases the mtDNA level and membrane potential
Talebi *et al.*, 2012; Zhang *et al.*, 2013	Alpha-lipoic acid	Improves in-vitro follicle development Decreases follicular ROS production Improves oocyte maturation rates Enhances the antioxidant ability of oocytes

[a] This was the only clinical trial in humans. It was discontinued before recruiting enough patients to achieve statistical power due to proof of negative effects on embryogenesis associated with polar body biopsy, such as higher rates of embryonic cleavage arrest and fragmentation. The remaining evidence listed in the table was obtained in animal-based models.

Modern mitochondrial therapies

The transfer of ooplasm from young donor oocytes to patient oocytes emerged as an innovative therapeutic approach in the 1990s to overcome poor embryonic development in patients with multiple IVF failure (Cohen et al., 1997, 1998). However, a prospective follow-up showed that this practice may change the pattern of mtDNA transmission, resulting in a persistent heteroplasmic mitochondrial population with the theoretical risk of mitochondrial disease inheritance (Barritt et al., 2001a; Brenner et al., 2000). In addition, cases of Turner's syndrome and autism have been described (Barritt et al., 2001b, 2001c). In 2001, the Food and Drug Administration (FDA) suspended the use of this technique because of ethical and biological concerns (Zoon, 2001).

As a result, new alternatives have been proposed. Those with the best expectations include mature oocyte chromosomal spindle transfer or pronuclear transfer, both of which are classified as nuclear genome transfer techniques (Craven et al., 2010; Tachibana et al., 2013). When these approaches are used as mitochondrial replacement therapies (MRT), they exhibit mtDNA carryover and heteroplasmy rates of <2% (Burgstaller et al., 2015; Cree and Loi, 2015; Wolf et al., 2015). However, stem cells from MRT embryos have been demonstrated to suffer a progressive loss of donor mtDNA, culminating in

an important reversal to the maternal haplotype in a few cases (Kang et al., 2016; Yamada et al., 2016). The exact mechanism causing such a reversion phenomenon remains obscure. Moreover, although the carryover rate is low, the mtDNA mutation load can reach higher frequencies because of vegetative segregation, relaxed replication, or even based on the mtDNA genetic bottleneck hypothesis (Stewart and Chinnery, 2015). Importantly, results obtained from in-vitro studies may not precisely represent mtDNA clonality behaviour *in vivo*.

Interestingly, the UK Parliament was the first to vote for mitochondrial donation, early in 2015. In 2016, the Human Fertilization and Embryology Authority (HFEA) endorsed this decision in a public statement. Therefore, the UK became the first region in which MRT can be legally performed in the clinical context of relevant mitochondrial diseases. Many countries have no specific federal legislation regarding the use of such techniques, and in other places they are strictly prohibited (Schandera and Mackey, 2016).

The global scientific community long thought that women are born with a limited number of ovarian follicles and oocytes, which are utilized during the reproductive period and depleted by menopause. In 2004, Johnson et al. (2004) reported for the first time the existence of mitotically active cells in mouse ovaries that express cell markers identifying them as oogonial germline stem cells. Later research by the same group demonstrated that these egg precursor cells also exist in human ovarian tissue, which led to a patent in 2011 by Tilly and Johnson (US Patent number US7955846). Many subsequent studies could not verify these findings, and a large number of authors remain sceptical (Begum et al., 2008; Byskov et al., 2011; Ghazal, 2013; Gosden and Johnson, 2016).

Nevertheless, the possibility of autologous mitochondrion transplantation was considered by the same group as a therapeutic option for women with a history of multiple IVF failures, coining the term autologous germline mitochondrial energy transfer (AUGMENT) (Tilly and Sinclair, 2013). AUGMENT consists of obtaining ovarian cortical fragments via minimally invasive laparoscopy, identifying and isolating oogonial precursor cells, isolating a functional mitochondria population from oogonial precursor cells, and transferring these mitochondria into the oocyte during the intracytoplasmic sperm injection (Woods and Tilly, 2015). Not only has this technique not yet been approved by the FDA in the USA, but detailed information on the protocol for harvesting these cells is lacking, which jeopardizes AUGMENT's credibility (Gosden and Johnson, 2016).

In a well-designed animal model, pig oocytes presenting mtDNA deficiency were supplemented with autologous mitochondrial isolates from metaphase II oocytes, achieving a significantly higher blastocyst rate and a substantial improvement in mtDNA replication during embryogenesis (Cagnone et al., 2016). Notably, neither pregnancy rates nor reproductive outcomes were assessed in this study, and oocytes were matured in vitro. Furthermore, despite using AUGMENT's concept of autologous mitochondrial transplantation, mitochondria were not isolated from oogonial stem cells while performing these experiments.

In humans, some preliminary reports have shown increased clinical pregnancy rates using AUGMENT in women with very poor ART outcomes. These were prospective cohort studies with historical controls and prone to regression toward the mean. Importantly, the treatment was not helpful for older patients. Most of the patients included in the completed trials were younger than 40 years of age and, as such, were also those with the best reproductive outcomes. Among the 16 women aged between 41 and 45 years, only one patient had an ongoing clinical pregnancy, and there were no pregnancies in the four patients aged over 46 (Fakih et al., 2015; Oktay et al., 2015). It is fair to say that these studies are preliminary at best, and it is too early to claim any clinical utility of this approach.

Future perspectives and conclusions

Embryo selection is critical for the success of ART programmes. The morphological and morphometric embryonic scoring system is the most widely used tool. However, this method's accuracy in predicting embryo quality is low, as at least 40% of embryos with a normal morphology may have some type of chromosomal disorder (Tsai et al., 2010). A review of 15,169 comprehensive chromosomal screenings found an aneuploidy prevalence of more than 40% in young women, and rates as high as 88% at the age of 44 (Franasiak et al., 2014).

To improve infertility treatment outcomes, new tools for assessing embryo viability must be developed. Certainly, new insights will arise from research focusing not only on mitochondrial function, but also on molecular analysis of follicular fluid, granulosa and cumulus cells, oocytes, and embryos.

Although the AUGMENT approach has been claimed as an alternative for women with very poor ART outcomes (Woods and Tilly, 2015), many questions remain unanswered. Mainly, worries have been raised about the mitochondria quantity and quality of oogonial stem cells along with its real potential to improve oocyte quality. Notably, this procedure is not useful in the case of pathologies related to mtDNA mutations, known as mitochondrial diseases, which rely on MRT, also referred to as mitochondrial donation, mitochondrial transplantation and mitochondrial transfer.

The first live birth derived from oocyte spindle transfer in 2016 brought us hope concerning modern mitochondrial therapies (Zhang et al., 2017). Despite several methodological limitations discussed elsewhere (Alikani et al., 2017), it represents a decisive advance and exciting trend in the field of reproductive medicine. Although the mtDNA carryover rate using MRT is low, lifetime changes in mtDNA heteroplasmy level and its consequences have not been sufficiently studied.

Certainly, these new approaches require long-term follow-up studies to establish both the safety of genome manipulation and potential consequences related to epigenetic modifications. It is equally important that ethical aspects be discussed extensively, and technical improvements have yet to be revealed. Soon, we may debate the use of genome manipulation not only for mitochondrial diseases, but also as an alternative to oocyte donation for couples with nuclear DNA mutations or RNA-related diseases, as recently accomplished through remarkable in-vitro experiments (Batra et al., 2017; Ma et al., 2017).

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