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Aging and male reproductive function: A mitochondrial perspective

Sandra Amaral[1], Alexandra Amaral[1], Joao Ramalho-Santos[1,2]

[1]CNC Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, [2]Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal

TABLE OF CONTENTS

1. ABSTRACT

Researching the effects of aging in the male reproductive system is not trivial. Not only are multiple changes at molecular, cellular and endocrine levels involved, but any findings must be discussed with variable individual characteristics, as well as with lifestyle and environmental factors. Age-related changes in the reproductive system include any aspect of reproductive function, from deregulation of the hypothalamic-pituitary-gonadal axis and of local auto/paracrine interactions, to effects on testicular stem cells, defects in testicular architecture and spermatogenesis, or sperm with decreased functionality. Several theories place mitochondria at the hub of cellular events related to aging, namely regarding the accumulation of oxidative damage to cells and tissues, a process in which these organelles play a prominent role, although alternative theories have also emerged. However, oxidative stress is not the only process involved in mitochondrial-related aging; mitochondrial energy metabolism, changes in mitochondrial DNA or in mitochondrial-dependent testosterone production are also important. Crucially, all these issues are likely interdependent. We will review evidence that suggests that mitochondria constitute a common link between aging and fertility loss.

2. AGING

Aging is, and has always been, a major social concern. In the last century we have witnessed a demographic trend towards increased longevity, which is reflected in an increasingly elderly population. Nevertheless, differences in the lifespan of males and females have been observed across species, with females tending to live longer (1). Therefore, is not surprising that scientists involved in biogereontological research keep joining efforts in an attempt to better understand the complex riddle of molecular and biochemical mechanisms underlying aging, and possibly to find targets of intervention to revert the process, or at least diminish its effects. While for over 70 years it has been established that dietary restriction (DR) extends lifespan in several organisms, in the last two decades a variety of results demonstrated that longevity could be extended through the manipulation of individual genes in model organisms (2).

Aging can be defined as a complex multifactorial phenomenon characterized by a time-dependent general decay of physiological functions of an organism, associated with an increasing risk of morbidity and mortality ultimately mirrored in lifespan, and in which a complex array of genetic and environmental factors plays an important modulating role (3-5).

The driving force for aging processes seems to be stochastic damage, inflicted firstly to cells and their components as well as to the extracellular matrix (4,6). However, not all cells are equivalent in this aspect. In fact, cellular manifestations of aging are more pronounced in long-lived post-mitotic cells (such as neurons and cardiac myocytes), than in proliferating cells (such as stem and progenitor cells) and in the short-lived post-mitotic cells that develop from them. This can be explained by the strict differences between these cell types. While non-dividing cells are unable to maintain their structure and functionality over time, contributing to the degeneration of the whole organism, dividing cells remain highly functional, even at the end of a normal lifespan, probably due to the existence of a constant and renewable pool of stem and progenitor cells. In contrast, long-lived post mitotic cells are only rarely replenished from stem cells, a fact that can determine some of the profound alterations brought about by age (4,6,7). Nonetheless short-lived post-mitotic cells were also suggested to suffer from aging-related phenomeana that are probably due to changes in stem and progenitor cells. In fact it had been recently shown that the proliferation potential of stem and progenitor cells declines with age (7). Accordingly, Ratajczak and coworkers have also suggested that adult tissues are endowed with a pool of very small embryonic-like stem cells that ensure tissue maintenance during lifetime and that this pool decreases in an age-dependent manner, being negatively regulated by signaling pathways already proven to decrease lifespan (such as the insulin/IGF pathway) (8).

Overall cellular aging can manifest itself in a variety of ways. For example, aged cells are often larger than younger counterparts, a situation that may result from an excess of accumulated "waste" material, also resulting in progressive degeneration. On the other hand nuclei of aged cells are distinguishable for having increased levels of heterochromatin, as well as damage to both DNA and nuclear proteins. This damage is also found in proteins and lipids both in the cytoplasm and plasma membrane, in the latter case leading to changes in membrane fluidity and consequent disturbances in molecular transport, membrane permeability, and intracellular signaling. The accumulation of aberrant proteins within aged cells mirrors reactive oxygen species (ROS)-induced damage and incomplete digestion of proteins that have been modified by exposure to ROS. However, the most notable features observed in aging cells are changes to mitochondria. Senescent mitochondria exhibit structural deterioration, ranging from swelling and loss of cristae to complete destruction and homogenization of the matrix and mitochondrial membranes, resulting in the formation of amorphous material. These senescent mitochondria are frequently enlarged, the reason why they are sometimes called "giant" mitochondria. Mutations in the mitochondrial genome (mtDNA) and changes to mitochondrial proteins also progressively increase with age (6,7).

In the last 20 years many molecular mechanisms have been proposed to explain the effects of aging as a result of research mainly focused on two concepts. The first postulates that certain genes control lifespan through modulation of hormone secretion and associated signalling pathways, and the second is based on the assumption that accumulation of oxidative damage is responsible for the progressive functional decline with advancing age (3). However, and noteworthy, these two pathways are probably more interconnected than originally anticipated.

3. MITOCHONDRIA AND AGING: THE OXIDATIVE STRESS CONNECTION?

Mitochondria are thought to be at the hub of cellular events during the aging process, although recent alternatives have called these theories into question (for review see 9-12). Early in 1956 Harman proposed that free radicals produced during normal metabolism cause cumulative oxidative damage that contribute to physiological decline and ultimately results in aging and death, the so-called *Free radical theory of aging* (13). Later, the same author extended the original idea, identifying mitochondria as the main producers and also the major target of oxidative damage, a situation that implies a vicious cycle in which damaged mitochondria produced increased amounts of ROS, in turn leading to a progressive accumulation of damage that culminates in the process of aging. This is usually called the *Mitochondrial theory of aging* (14). As a result of intense research, Harman's original hypothesis has then been polished in order to address the role of all forms of ROS, and is now referred as the *Oxidative stress theory of aging* (12). For more than 50 years several studies have focused on the putative link between oxidative stress, longevity and aging, making this theory one of the major aging hypothesis (for review see 12,15-17). More recently, the *Mitochondrial-Lysosomal Axis Theory* has again involved mitochondria in the process of aging (7).

However, although correlations between oxidative stress and aging are evident, the exact causal nature of these correlations is disputed. Alternative theories either question the validity on oxidative damage accumulation with aging, or link it with an increased propensity for degenerative disorders (e.g. cancer, neurodegenerative diseases, etc) that, although related with a diminished lifespan and with the quality of life, are not necessarily directly involved with aging itself (for review see 9-12,18,19). Also, the procedures (including the use of isolated mitochondria) and/or the specific cell types used (long lived post-mitotic cells or other cell types) have also to be kept in mind when the data obtained in these kinds of studies is analysed (7,10), However, it is also important to note that the oxidative stress theory does not exclude that other factors may also be involved in the aging process. Still, recent studies continue to produce results in favour of the *Oxidative stress theory of aging* (20-22). Clearly the scenario is more complex than predicted by initial hypotheses, and several new players are frequently being suggested, as is the case of sirtuins and p53 (21,23), although their true role on the aging process is debated (24)**.** It seems clear that a multifactorial approach must be acknowledged in future studies, as it is extremely unlikely that one sole line of research will provide conclusive results in this field. At any rate we will discuss bellow some of the

already known and established aspects that are entailed in the aging-mitochondria-oxidative stress triad.

It has been shown that mitochondrial oxidation is the major source of oxidative lesions that accumulate with age, although there are other cellular enzyme systems capable of generating ROS. The general belief is that ROS are produced as by-products of the electron transfer chain (ETC), mainly at complex I and III (25). Nonetheless, it was recently demonstrated that ROS are also produced by mitochondrial NADPH oxidases such as NADPH oxidase 4 (NOX4), the expression of which is also up regulated by aging in the heart (26). As the major intracellular source of ROS, mitochondria are also vulnerable to direct attack by ROS. Under normal conditions ROS are scavenged by a complex set of enzymatic and non-enzymatic antioxidants, but several parameters, including aging, seem to affect this fine equilibrium. The role of antioxidants in the aging process is controversial and has been the target of extensive research, including studies in knockout (KO) and transgenic (TG) mice. However results have been inconsistent, with reports of both positive and negative or no correlations with lifespan, once again putting the oxidative theory of aging in question (9,11,12,19,22). However, new antioxidants continue to be identified and will probably lead to further insights (9, 23, 27).

3.1. Age-related oxidative damage to mitochondrial macromolecules and bioenergetics

The levels of ROS-modified proteins and lipid peroxides in mitochondria seem to increase with age (28,29). To complicate this scenario, mitochondrial membrane lipids, particularly cardiolipin in the inner mitochondrial membrane, are particularly at risk of oxidative damage, due to high levels of polyunsaturated fatty acid (PUFA). Since cardiolipin content plays a pivotal role in mitochondrial membrane function it is therefore essential for mitochondrial bioenergetics. In fact, cardiolipin content was observed to decrease with age in several tissues and thus it is not surprising that oxidative stress and aging are associated with decreased activities of inner membrane proteins, which in turn are also susceptible to oxidative damage (30,31). Other components of the oxidative system, such as the adenine nucleotide transporter (ANT), ATP synthase, and the matrix enzyme aconitase are also highly susceptible to oxidative stress and, if affected, will negatively impact mitochondrial functionality (32-34). The transport of metabolites to and from mitochondria is also influenced by aging, as is the case of malate import, which has been demonstrated to decrease with age (35), with cardiolopin-related issues likely playing a role in the process. Furthermore, mtDNA is near the ETC where ROS production occurs, and is therefore an easy target for ROS-induced damage (36-38). However several considerations should be kept in mind when interpreting data relative to mtDNA and its increased sensitivity to oxidative stress (for discussion see11, 38).

Regardless, ETC-induced ROS damage to mtDNA seems to accumulate during the lifetime of a cell or organism, and may be a result of deficient mitochondrial biogenesis and/or turnover (7,15,39). Additionally, defects in mtDNA accumulated during aging may also reduce or prevent the renewal of mitochondrial proteins (7,15). Damage to mtDNA and mtDNA mutations can be propagated by both mitochondrial and cell divisions and this is expected to lead to a general decline in respiratory capacity, impairing ATP production and augmenting ROS production, thus resulting in an overall increase in mtDNA mutations and oxidative stress, again creating a vicious cycle (38,39). In addition, age-related mitochondrial oxidative stress seems to have a key role in promoting the intrinsic pathway for apoptosis, where it is considered an early event. In fact, aging and apoptosis share many features, such as decreased mitochondrial membrane potential, higher lipoperoxide levels, glutathione oxidation, and mtDNA oxidative damage, mainly due to an increase in oxidative stress common to both processes (17,38,40).

All the above-mentioned factors contribute to a reported gradual decline in mitochondrial respiratory function with age, both in humans and in laboratory animals. Respiratory control, phosphorylative efficiency, the rates of resting (State 4) and ADP-stimulated (State 3) respiration, as well as the activities of respiratory enzyme complexes decrease with age in human tissues (25,38), although again it should be noted that some data has been called into question, or alternatively interpreted (9-12). Regardless, aging was also associated with decreased expression of the LON protease (involved in mitochondrial biogenesis), ATP synthase subunits, NADP transhydrogenase, and with decreased expression of genes involved in fatty acids and cholesterol synthesis, as well as those involved in protein turnover (15,16,38). Additionally, decreased ATP synthesis due to an age-dependent mitochondrial dysfunction can potentially lead to an energy crisis that would affect several energy-dependent cellular processes (15).

3.2. Uncoupling proteins, reactive oxygen species and aging

The proton motive force set up across the inner membrane also influences mitochondrial ROS production. In fact, mild uncoupling caused by activation of uncoupling proteins (UCPs) might lower the proton motive force, attenuate mitochondrial ROS production, and protect against ROS-related cellular damage (41). Several studies have examined the effects of UCP deletion or overexpression on aging and lifespan. Fridell *et al* (2005) showed that *Drosophila* lifespan can be lengthened by approximately 10-30% via the overexpression of human UCP2 in the nervous system, and that the production of ROS was reduced in hUCP2 expressing flies (42). On the other hand, the same researchers described that UCP5 knockout flies live longer in low caloric diets, but had reduced survival during starvation (43). In the same vein, the deletion of UCP4-like protein in the nematode *C. elegans* resulted in increased ATP levels but had no effect on lifespan when compared to controls (44). In addition, some studies in rodents also provide evidence in terms of a possible role of UCPs on lifespan and aging. For instance, an age-dependent increase in proton leak rate and a decrease in ATP turnover reactions were observed in mouse hepatocytes (45). Moreover, skeletal muscle

respiratory uncoupling due to UCP1 expression seems to diminish age-related disease in mouse models (46) and the overexpression of UCP3 in rats blunts age-induced increase in ROS formation (47). Additionally, and although the effects on lifespan were not reported, it was also shown that UCP1 ablation reduces the mitochondrial superoxide production in brown adipose tissue, probably through a repression of cold stimulated substrate oxidation (48). In a different study the effect of different diets on lifespan was assessed in wild type and in transgenic mice with increased skeletal muscle UCP1, and it was observed that mitochondrial uncoupling alleviated the detrimental effects of high-fat diets, contributing to an increase in longevity (49).

3.3. Imperfect (mitochondrial) turnover and aging

Oxidative stress impacts not only mitochondria but also extra-mitochondrial biomolecules, including lipids, proteins and DNA (50-52). As already mentioned, aging is characterized by the accumulation of damaged proteins, lipids and dysfunctional mitochondria in post-mitotic cells, suggesting aging-related changes in degradation pathways (6,7). Cells are endowed with several mechanisms to degrade damaged components, including those resulting from oxidative stress. In fact, autophagy, a normal and well-organized process that provides intralysosomal degradation of cell components, has been demonstrated to be downregulated in aged cells. Autophagy downregulation would lengthen the half-lives of long lived proteins, lipids and organelles leading to a situation where cells would be forced to perform their functions under sub-optimal conditions (6,7).

Autophagy can also be a specific mechanism to eliminate severely damaged mitochondria, a process dubbed mitophagy. Attenuation of the effects of mitochondrial damage can occur either by mitophagy or via the mitochondrial proteolytic machinery, composed by ATP-dependent matrix proteases such as LON, Clp-like and AAA proteases. In line with previous discussion the activity of these enzymes seems to be decreased in old rats (7). A decreased autophagic capacity in aged cells, together with alterations in the mitochondrial proteolytic machinery, will thus lead to a decline in mitochondrial turnover and accumulation of damage with age. Senescent mitochondria will gradually produce less ATP and more ROS, resulting in enhanced oxidative stress, hindering mitochondrial turnover even more and possibly inducing apoptotic cell death (6,7). Conversely, it also important to note that uncontrolled autophagy can lead to apoptotic or necrotic cell death, apparently through common regulators such as Bcl-2 family members. However, this is still a debated issue and we cannot say for sure which is the relative contribution of these opposing effects to cell death and aging (for review see 161, 162, 163). Additionally, as nonfunctional mitochondria are eliminated by autophagy, mitochondrial biogenesis is necessary to maintain energy production and cellular homeostasis. This process has also been shown to be decreased with age, with observable changes in many of the factors involved. (7). The balance between fusion and fission is central to the maintenance of mitochondrial dynamics, including morphology, distribution, function and inheritance. When this fine balance is disturbed at any level mitochondrial and cellular function will be further compromised. In fact, components of the fusion/fission machinery, such OPA1 or Drp1, are also prone to alterations with aging. While changes in the fusion machinery will not allow the dilution of damage between normal and senescent mitochondria, disturbance in the fission machinery can have consequences in terms of the elimination of damaged mitochondria (for review see 7).

In conclusion, several lines of evidence suggest that mitochondrial function may be impaired in aging, as judged by a decline in bioenergetic parameters, an increase in peroxide production and mitochondrial size, alterations in mtDNA, a decrease in mitochondrial protein synthesis and in the expression of genes involved in mitochondrial turnover (15,17).

4. THE MALE REPRODUCTIVE SYSTEM

The primary sex organs of the male reproductive system are the two testes in which sperm are produced (53,54). The testis contains seminiferous tubules that consist of germinal epithelium and peritubular tissue (*lamina propria*) (54,55). The epithelium contains two basic cell types: somatic and germinal cells (56). Germ cells are at different developmental stages, including spermatogonial stem cells (SSCs, the self-renewing stem cells of the testis) and differentiated cells formed during and following meiosis, namely primary and secondary spermatocytes and spermatids. These cells are located within invaginations of somatic Sertoli cells, with which they maintain an intimate and cooperative relationship (55,56). Sertoli cells form the blood-testis barrier, creating a separate and immunoprevileged site in the testis. Other Sertoli cell functions include germ cell mechanical and nutritive support, phagocytosis, participation in spermiation, secretion of testicular fluid for sperm transport, production of endocrine and paracrine substances that regulate spermatogenesis, and secretion of androgen binding protein (ABP) (55,57). Testosterone-secreting Leydig cells are found in the intertubular tissue surrounding the capillaries and have a prominent role in the maintenance of spermatogenesis, the differentiation of male sexual organs and male secondary sex characteristics.

Spermatogenesis takes place in the seminiferous tubules and is a highly dynamic and metabolically active biological process during which haploid spermatozoa are produced through a gradual transformation of an interdependent population of germ cells. These cells sequentially migrate from the basal compartment towards the luminal regions of the tubules, passing the blood-testis barrier. Spermatogenesis can be divided in three main phases (Figure 1) and its duration is species-specific (55,56).

Accurate spermatogenesis is dependent on several hormonal messengers acting through endocrine, paracrine, and autocrine pathways. Normal male reproductive function depends on the pulsatile secretion of

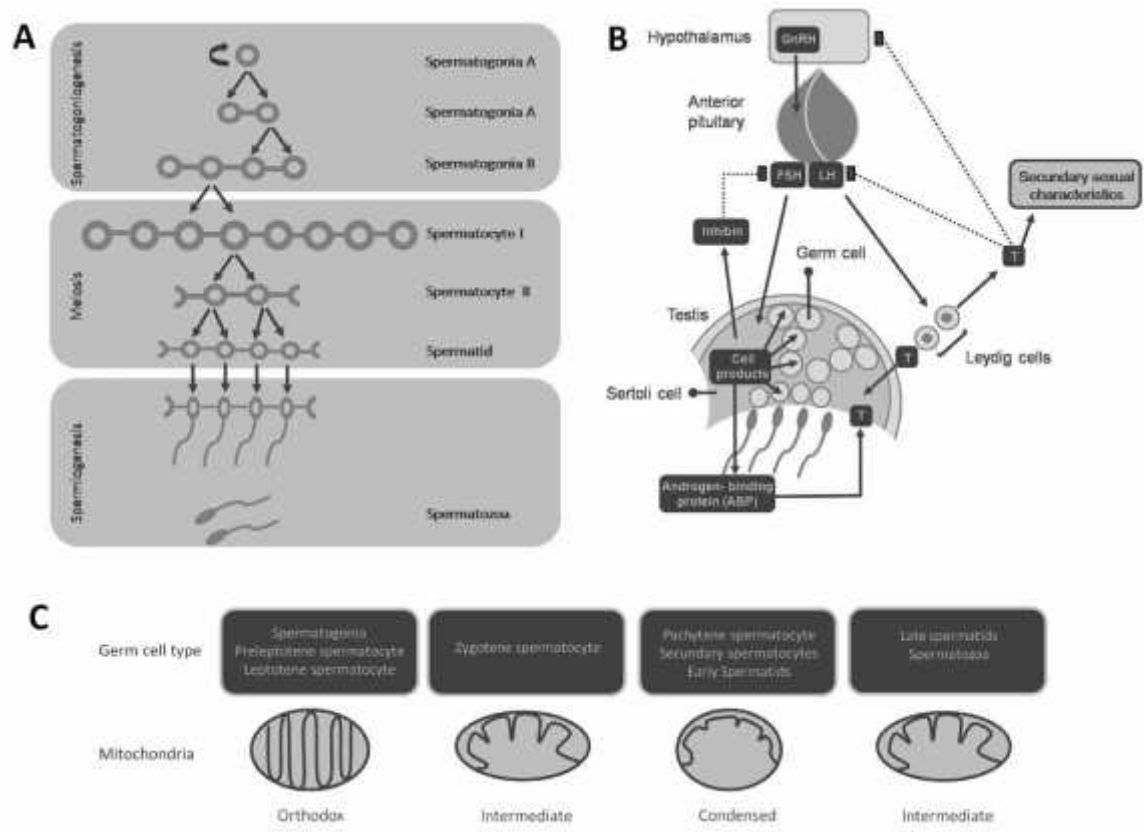

Figure 1. Spermatogenesis and its hormonal regulation. Spermatogenesis (A) is the process culminating in the production of haploid sperm. It can be subdivided in three major phases. Spermatogoniogenesis is characterized by spermatogonial proliferation and is followed by the formation of primary spermatocytes at the start of meiosis, a long phase characterized by changes in chromatin. After two cell divisions round spermatids are formed and suffer terminal differentiation in the phase of spermiogenesis to produce spermatozoa that leave the testis during spermiation. This process is highly regulated (B). The male hypothalamus secretes GnRH that stimulates the pituitary to produce FSH and LH. FSH exerts its effects on the testis by stimulating Sertoli cells to secrete factors such as inhibin, which exerts a negative feedback on the pituitary. FSH is important for the maturation of germ cells. LH stimulates Leydig cells to produce testosterone, which has a key role in spermatogenesis and expression of male secondary characteristics. Additionally testosterone can provide a negative feedback to the pituitary reducing LH secretion (adapted from 53). (C) The mitochondria of germ cells modify their morphology, number and localization during spermatogenesis and three types of mitochondria can be recognized.

luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary, under the influence of gonadotrophin releasing hormone (GnRH) released by the hypothalamus. Pulsatile LH stimulates Leydig cells to produce testosterone, which in turn exerts a negative feedback on GnRH and gonadotrophin secretion. In turn, FSH stimulates Sertoli cell proliferation, a necessary step for the maturation of germ cells, given that Sertoli cell number largely determines the number of germ cells that can be correctly nurtured in the testis. In fact, FSH and testosterone act co-operatively at several points during spermatogenesis. Additionally, Sertoli cells *per se* secrete inhibin in a feedback mechanism targeted to the hypophysis (55,58,59) (Figure 1).

4.1. Mitochondria and male reproductive function

Early in development male gonads have a higher energy requirement than ovaries (60). Additionally in SSCs the number of active mitochondria seems to vary with the development stage (61). Furthermore, the existence of numerous mitochondria in male germ cells highlights their importance in testicular metabolism (62).

In the adult testis survival of germ cells is strictly dependent on carbohydrate metabolism, including both glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). However different cells in the seminiferous epithelium utilize distinct substrates (63,64). In fact, during spermatogenesis, there is a considerable change in the energy metabolism of germ cells, mainly due to the blood testis barrier and the subsequent changes to the surrounding medium. Therefore, germ cells develop in a tubular *milieu* that is highly controlled by Sertoli cells (65,66). It has been previously described that spermatogonia (SSCs), mature sperm and the somatic Sertoli cells exhibit high glycolytic activity, whereas spermatocytes and spermatids produce ATP mainly by OXPHOS (63,64,67-70). Overall, this could merely be a question of opportunity, but not necessarily in

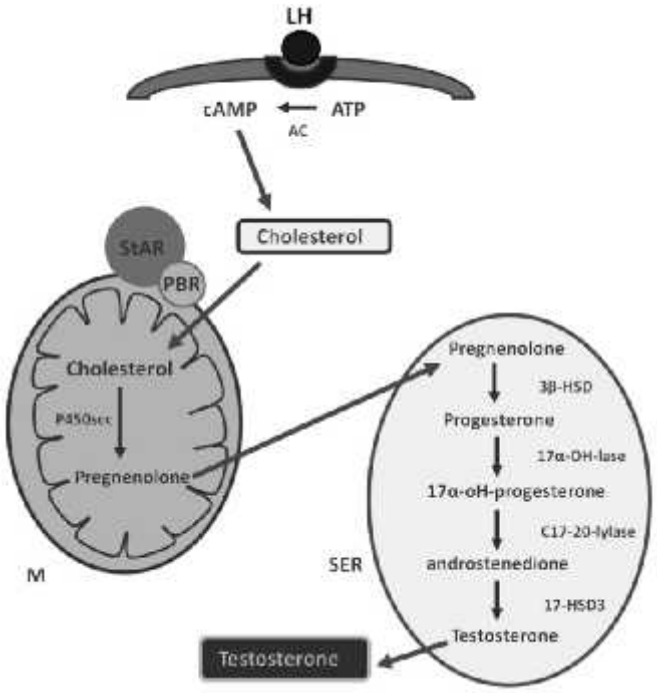

Figure 2. Leydig Cell Steroidogenesis. After LH binds to a plasma membrane receptor in Leydig cells, a signaling cascade is initiated, including activation of Adenyl cyclase (AC) and increased production of cAMP, cholesterol transport into the mitochondria facilitated by StAR protein and peripheral benzodiazepine receptor (PBR). Once in the mitochondria (M), cholesterol will be converted to pregnenolone through the action of side chain cleavage cytochrome P450. Pregnenolone then diffuses to the smooth endoplasmatic reticulum (SER) where it is further metabolized via the action of 3β-hydroxysteroid dehydrogenase $\Delta 5$-$\Delta 4$-isomerase (3β-HSD) to progesterone. Progesterone in turn is converted by a two step process, involving17α-hydrosylase (17α-OH-lase) and C17-20 lyase, to androstenedione that is finally converted to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase type III (17 HSD3).

all cases. For example, even though spermatocytes have the machinery to produce energy through glycolysis, they rely mostly on lactate, probably because the testicular fluid is rich in lactate and poor in glucose. In the same vein, being closer to the seminiferous tubule wall, spermatogonia have facilitated access to oxygen (supplied by the blood vessel surrounding the tubules and that only reach the lumen by diffusion), and are thus expected to use OXPHOS instead of glycolysis. Similarly, spermatocytes with less access to oxygen were expected to utilize glycolysis, however this had been proven not to be the case. Even in the case of sperm it is still a matter of debate if they obtain energy exclusively via the glycolytic pathway, or if OXPHOS is also involved (71). Overall, it seems that substrate availability imposed by seminiferous tubules compartmentalization, together with ATP demand, may prime the cells to different adaptations.

Nonetheless, and contributing towards the understanding of testicular metabolism, our group had shown that testicular mitochondria are quite particular, presenting different bioenergetic parameters when compared to those seen in mitochondria from other tissues. In fact, testis mitochondria seem to consume less oxygen to generate approximately the same maximum electric potential as mitochondria from other organs (72,73).

Three types of mitochondria are identified during spermatogenesis: the usual orthodox-type mitochondria in Sertoli cells, spermatogonia, preleptotene and leptotene spermatocytes; the intermediate form in zygotene spermatocytes; and the condensed form in pachytene spermatocytes, secondary spermatocytes and early spermatids, a conformation that shifts back to the intermediate form in late spermatids and sperm (56, 58) (Figure 1). These structural changes mirror the metabolic dynamics during spermatogenesis (62,74) and can also be induced by factors released by Sertoli cells (75).

In parallel with the described structural changes, several mitochondrial proteins, such as heat shock protein (hsp) 60 and 70, Lon protease and sulphidryl oxidase (SOx), are known to be differentially expressed during distinct phases of spermatogenesis (62). Additionally, there are several testis-specific proteins including, for instance, cytochrome c and the subunit VIb-2 of cytochrome c oxidase (COX) (reviewed in 71).

The physiological death of germ cells via apoptosis (mainly at the first wave of spermatogenesis) seems to be a continuous event in the spermatogenic process, limiting the number of germ cells that reach maturity, and can be increased by various stimuli (hormone deprivation, heat, toxin exposure) (55). Several works have

described the participation of the mitochondrial pathway in this process, namely showing altered levels of cytochrome c release, Bax and Bcl-2 in an array of both physiological and induced conditions (reviewed in 71).

Furthermore, mitochondria play a major role in Leydig cell sterodoigenesis, a process that involves the steroidogenic acute regulatory (StAR) protein-mediated delivery of free cholesterol to the inner membrane of mitochondria (Figure 2). Once there it is then converted to testosterone through a series of steps (76). Energized, polarized, and actively respiring mitochondria have been proven to be necessary for sterodoigenesis in Leydig cells, in several studies using different mitochondrial inhibitors (77,78).

4.2. Mitochondria, oxidative stress and male infertility

Oxidative stress is a common pathology seen in approximately half the population of infertile men. There are two main sources of ROS in semen: leucocytes and sperm. Although the production of ROS by sperm plays a positive role in fertilization at low levels (namely in capacitation, a maturation process required for the acquisition of fertilization ability), when produced at high levels it can lead to potential toxic effects on sperm quality and function (79,80). Additionally, environmental and lifestyle factors as well as pathologies of the reproductive system and chronic diseases are sources of sperm oxidative damage (80). Although seminal plasma contains an ample array of protective antioxidants such as Superoxide dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX), these defenses are less abundant in sperm and seem to be impaired in cases of male infertility (79,80).

ROS is thought to influence fertility by affecting both sperm membranes and sperm DNA. The first mechanism reduces sperm motility and its ability to fuse with the oocyte, while the latter compromises paternal genomic contribution to the embryo (for review see 80). In fact, similarly to what occurs in the inner mitochondrial membrane, sperm are especially vulnerable to oxidative stress- induced damage due to the high portion of PUFAs, and also due to the low concentrations of scavenging enzymes in their cytoplasm, both contributing to the defective sperm function observed in a high percentage of infertility patients (for review see 79).

However, and although the testis is endowed with several antioxidant enzymes, the overexpression or loss of which has serious implications on spermatogenesis, an effect of ROS early in spermatogenesis should not be excluded (81). There are several agents known to cause an increase in testicular oxidative stress, such as environmental toxins, or conditions such as varicocele, orchitis, cryptorchidism and aging, all of which leads to an increase in germ cells apoptosis and hypospermatogenesis (reviewed in 81). In fact, it has been observed that ROS-induced DNA damage may also potentiate germ cell apoptosis, leading to a decrease in sperm count, and thus to the decline of semen quality, both of which are associated with male infertility (79). Additionally, when large amounts of pathogenic mutant mtDNA accumulate in the testis the resulting mitochondrial respiratory dysfunction in spermatogenic cells leads to a decrease in energy production that ultimately induces meiotic arrest and abnormalities in sperm morphology, stressing the importance of mitochondrial respiratory function in mammalian spermatogenesis (82).

5. AGING AND MALE REPRODUCTION: ARE MITOCHONDRIA INVOLVED?

The reproductive decline during aging occurs in a species- and gender-specific manner. Whereas in women reproductive activity ends with the onset of menopause, in men this process is more gradual and men generally do not experience a complete cessation of reproductive capacity, and are capable of maintaining spermatogenesis until very late in life (83-86, 87). The reproductive decline in men develops as a combination of morphological changes in organs together with changes in endocrine networks. These characteristics are highly variable individually and seem to be influenced by lifestyle and environmental factors (83,84,88).

5.1. Changes in hormone levels and hypothalamus-pituitary-gonadal axis functionality

One of the most remarkable aspects of male reproductive aging is the gradual and progressive decline in testosterone levels. This decline begins about the age of 30 and decreases progressively as men age (89,90,91). Due to this reduction in testosterone levels, testosterone replacement therapy in older men has been the target of intensive research in the last years. However, the risk/benefit assessment is still a debated issue (84-86,92-94).

The age-related decline in serum testosterone may be explained by changes in any or in all of the components and regulatory levels of the hypothalamus-pituitary-gonadal axis (HPG). Impairment of testicular steroidogenesis at Leydig cells, as well as changes in other hormone production and in feedback mechanisms may be involved (83,84,88,91,95). FSH levels increase with age, and Sertoli cell-secreted inhibin (which exerts a feedback inhibition on FSH secretion) decreases with age (87). On the other hand, although LH levels show little age-related changes, these might have some significance. Compared with young men, healthy older men with low serum testosterone levels have an abnormal LH pulse frequency, reduced LH pulse amplitude, and more disorderly LH secretion, suggesting an age-associated impairment of the hypothalamic GnRH pulse generator (95-97). Androgen receptor expression in the hippocampus and the number of androgen binding sites in genital skin is also decreased in older men and, additionally, an age-related decrease in aromatase (a crucial enzyme in steroidogenesis) and estrogen receptor expression in the testis was also observed (98-100) Age-related hypothalamic pituitary dysfunction may also lead to a reduction in growth and thyroid hormone secretion and an increase in glucocorticoids, all of which will have a detrimental role in sterodoigenesis (reviewed in 91). Moreover, an age-related decrease of

dehydroepiandrosterone (DHEA), an adrenal precursor of estrogenic steroids, has also been observed with aging (101). In addition, the levels of many biological factors that influence sterodoigenesis are altered, including cytokines, interleukins, transforming growth factor-b1, tumor necrosis factor and ROS, all of which are increased in aged subjects (91).

5.2. Changes in testicular architecture

Altered testicular function and histomorphology also contribute to partial primary hypogonadism in elderly men, such as reduction in testicular perfusion, thickening of the *lamina propria* of the seminiferous tubules, decrease in Leydig cell number, increased accumulation of lipofuscin in Leydig cells (83,88), decreased number of LH receptors in the testis, compromised cholesterol transport and Leydig cell mitochondrial steroidogenesis, and a blunted rise in testosterone upon stimulation by human chorionic gonadotrophin (hCG) (reviewed in 83,88,102).

Other histomorphological changes observed in testis from older individuals include decreased number of spermatogonia, increased occurrence of multinucleated spermatogonia and giant spermatids, spermatogenesis arrest, accumulation of lipid inclusions and vacuolization in Sertoli cells, as well as a reduction in the number of these cells and a disruption of the blood testis barrier. Additionally, a disruption in communication between Sertoli and germ cells was also described (103) all contributing to impaired spermatogenesis in the aged testis (83,88,104). In support of these observations, Kimura and colleagues observed that the number of primary spermatocytes per Sertoli cell was decreased in aged testis due to accelerated apoptosis of these cells, contributing to spermatogenesis impairment. Conversely, the authors also found that apoptosis and proliferation were down-regulated in spermatogonia, suggesting that the decline in spermatogonial apoptosis might reflect a compensatory role for the diminished proliferation of these cells occurring during aging (105).

5.2.1. Stem cell aging

As already noted stem cells also age, despite their self-renewal properties. In the testis, SSC are located at the basal membrane and interact with Sertoli cells producing the stem cell niche, which need to be strictly regulated (106). Several studies have addressed the effects of aging on SSC and their niche. Ryu and coworkers used SSC transplantation to determine the effect of aging on testis stem cell/niche function in mice. They observed that male mice experienced a decrease in fertility, testis weight, spermatogenesis, and total stem cell content between 12 and 24 months of age. Interestingly, when stem cells were consecutively passaged at 3-month intervals to testes of young males, these stem cells continued to produce spermatogenesis. This suggests that infertility in old males results from deterioration of the SSC niche, not the stem cells themselves (107). Using a similar experiment design, Zhang and collaborators obtained different results pointing to the involvement of both SSC and somatic environment in the aging process (108). More recently, using a refined version of the same approach, it was demonstrated that both *in vivo* and *in vitro* aging negatively influence SSC function, as shown by the decrease in proliferation and shift to differentiation observed with aging, and including the identification putative genes that might have a role in SSC aging (109). Other authors reported a set of genes that were specifically expressed in an age dependent manner using isolated SSC from rats of different ages (110).

5.2.2. Sperm and age-associated male infertility

The true significance of aging on conventional semen parameters is controversial, and has been confounded by the different criteria and number of samples used in the studies performed so far. At any rate, the bulk of evidence suggests that increased age is associated with a decline in semen volume, sperm motility, and amount of morphologically normal sperm, but not with sperm concentration (111,112). Some studies also suggest a decrease in sperm viability, and in the seminal levels of both alpha-glucosidase and fructose (113,114). A few papers have analyzed the putative effects of aging in sperm functional parameters, which seems to be weak (115,116). Then again, contradictory data has been published, and sperm from older man were shown to have increased DNA fragmentation (see below), poor chromatin packaging (117) and altered kinematics (118). Given that changes in seminal parameters may not actually be functionally relevant for fertilization and early development, the relationship between male age and fertility status is even more difficult to establish, also because the end results are always influenced by the age of the female partner. Data on whether paternal age influences assisted reproduction techniques (ART) outcomes are conflicting (119,120). Interestingly though, and after analyzing a very large cohort, Matorras *et al* (2010) have shown that men around 35-39 years of age start to experience an exponential decrease in fertility, which is independent of the female partners age (121). Moreover, increased paternal age has been associated with pregnancy complications, and seems to have some risk for the offspring (112). For this reason, and although the negative effects of aging in male fertility develop gradually without an abrupt age threshold, semen donors in some countries have an upper age limit (< 40-45 years).

Indeed, both human and animal data suggest that fatherhood in aged males is associated with an increased risk for pregnancy loss or genetic disorders in the offspring, caused by sex chromosomal aneuploidies or structural sperm-bourne chromosomal abnormalities (122,123). Whether the well-documented increase in germline mutations during aging is a consequence of an accumulation of replication errors and/or of inefficient repair processes is not well understood. Paradoxically, it has been proposed that some of these mutations may confer a selective advantage to selfish spermatogonia (124,125). In either case, age-associated male germ cell genomic instability may be harmful to the offspring, causing neurodevelopmental diseases such as schizophrenia, autism and also rarer disorders such as achondroplasia and craniosynostosis. Furthermore, using a mouse model, it has recently been shown that the offspring of older males have an increased risk of *de novo* copy number variants (CNVs)

associated with altered behavioral and neurological phenotypes (126). Interestingly a positive correlation between paternal age and both sperm and offspring cells telomere length has also been shown (127,128), but the putative consequences of longer telomeres in the offspring are unknown. The mechanisms behind the age-dependent sperm telomere length elongation are also unclear, but may arise from epigenetic processes taking place in spermatogonial stem cells (SSC), or by the survival of a subgroup of SSC more resistant to aging (129).

Increasing evidence suggests that oxidative stress is at the origin of the above-mentioned age-dependent sperm defects. When compared to younger men, older men seem to have higher seminal ROS levels, increased sperm DNA damage (both single- and double-stranded DNA breaks), and more sperm with early apoptotic markers (130-132). Noteworthy, a positive correlation between age and ejaculate ROS levels was shown in a group of fertile men (133). Observations in Brown Norway rats also stress that the age-dependent decrease in sperm quality is associated with a reduced capacity to handle oxidative stress. In fact, the enzymatic activity of various antioxidant enzymes (such as glutathione peroxidases and superoxide dismutase) seems to be lower in sperm from older rats, which concomitantly have higher ROS production and higher lipid peroxidation when compared to sperm from younger counterparts (134). Interesting outcomes were also obtained by the comparative analysis of gene expression in the germ cells of this rat model. Specifically, it was shown that aging is associated with a differential regulation of DNA repair pathways, particularly with a decrease in base excision repair (BER) pathway, resulting in a deficient repair of 8-oxo-2'-deoxyguanosine lesions in germ cells and sperm (135).

5.3. The role of reproductive tissues in lifespan modulation

Although accepted for a long time, the idea that an investment in reproduction may shorten lifespan had been challenged by several recent studies. In fact, the somatic reproductive tissues in *C. elegans* have been shown to modulate lifespan. In the first classical experiments when germ cells were removed the animals lived longer. However when both germ cells and somatic reproductive tissues were removed the lifespan extension was no longer seen, suggesting that somatic gonad cells send life-extending signals to other tissues. Similar observations were also made in *Drosophila.* Several recent studies have focused on the pathways underlying these life-extending effects of somatic reproductive tissues. It seems that reproductive tissues exert influence in other tissues through signaling pathways such as the Insulin/IGF1 pathway (136). For instance, autophagy and lipolysis seem to have interdependent roles in the modulation of aging in this extended longevity model, suggesting that the maintenance of lipid homeostasis prolongs lifespan (137). Also interesting is the argument that higher reproductive success in the male (as well as in the female) may be associated with diminished immunity, and conversely, that both life expectancy and immunity are increasing at the cost of fertility (138).

5.4. Targets for intervention

Several studies have focused on possible strategies of intervention in order to attenuate the adverse effects of aging (and related oxidative stress) on the male reproductive system. Despite the already mentioned and controversial testosterone therapy, other interventions have been suggested.

It has been recently shown that taurine supplementation, noted (among other properties) for its antioxidant properties, could stimulate the secretion of LH and testosterone, elevate the levels of several antioxidant enzymes in the testis and improve sperm functionality in aged rats (139). Additionally, it has been reported that regular exercise protects the testis against age-related changes, an effect that is associated with decreased levels of oxidative damage to proteins, lipids and DNA in spermatogenic and Leydig cells (140). In 2008, Hamdem and coworkers observed that older rats treated with estrogens presented higher levels of antioxidant enzymes, decreased levels of lipid peroxidation and spermatogenesis recovery together with a higher expression of aromatase and estrogen receptors in the testis, suggesting a protective effect of estrogens in lowering oxidative stress (141). Lastly, caloric restriction (CR) is believed to be the most effective intervention to prevent chronic diseases and to slow the aging process (142). The male reproductive tract of the Brown Norway rat (which is extremely affected by aging) does not seem to be an exception. To this extent, data from Jervis and Robaire (2003) suggested that CR is able to attenuate or reverse age-related gene expression changes, improve biosynthetic capacities and reduce mitochondrial dysfunction in the aging epididymis (143). Using the same rodent model, these authors have also shown that long-term vitamin E treatment results in decreased expression of oxidative stress-related transcripts along the epididymis, whereas long-term vitamin E deficiency has exactly the opposite outcome, aggravating the effects of age on the accumulation of oxidative stress damage (144).

6. ARE MITOCHONDRIA A LINK BETWEEN AGING AND LOSS OF FERTILITY?

Several recent studies support the hypothesis that mitochondria are a common link between age and age-related loss of male fertility. In fact**,** analysis of testicular mitochondria has shown a decrease in mitochondrial function with age, including changes in fatty acid composition, possibly affecting fluidity and mitochondrial complex activity (145), correlated also with an increased production of superoxide anion, lipid peroxidation, and with reduced activity of antioxidant enzymes (146,147). The balance between pro- and anti-oxidative agents seems also to be altered in aging testis mitochondria, particularly with a shift in the glutathione redox state towards the pro-oxidizing condition (148).

Recently, we have examined the effects of age on the bioenergetic characteristics of testicular mitochondria isolated from rats of different ages. We observed that mitochondrial respiratory and

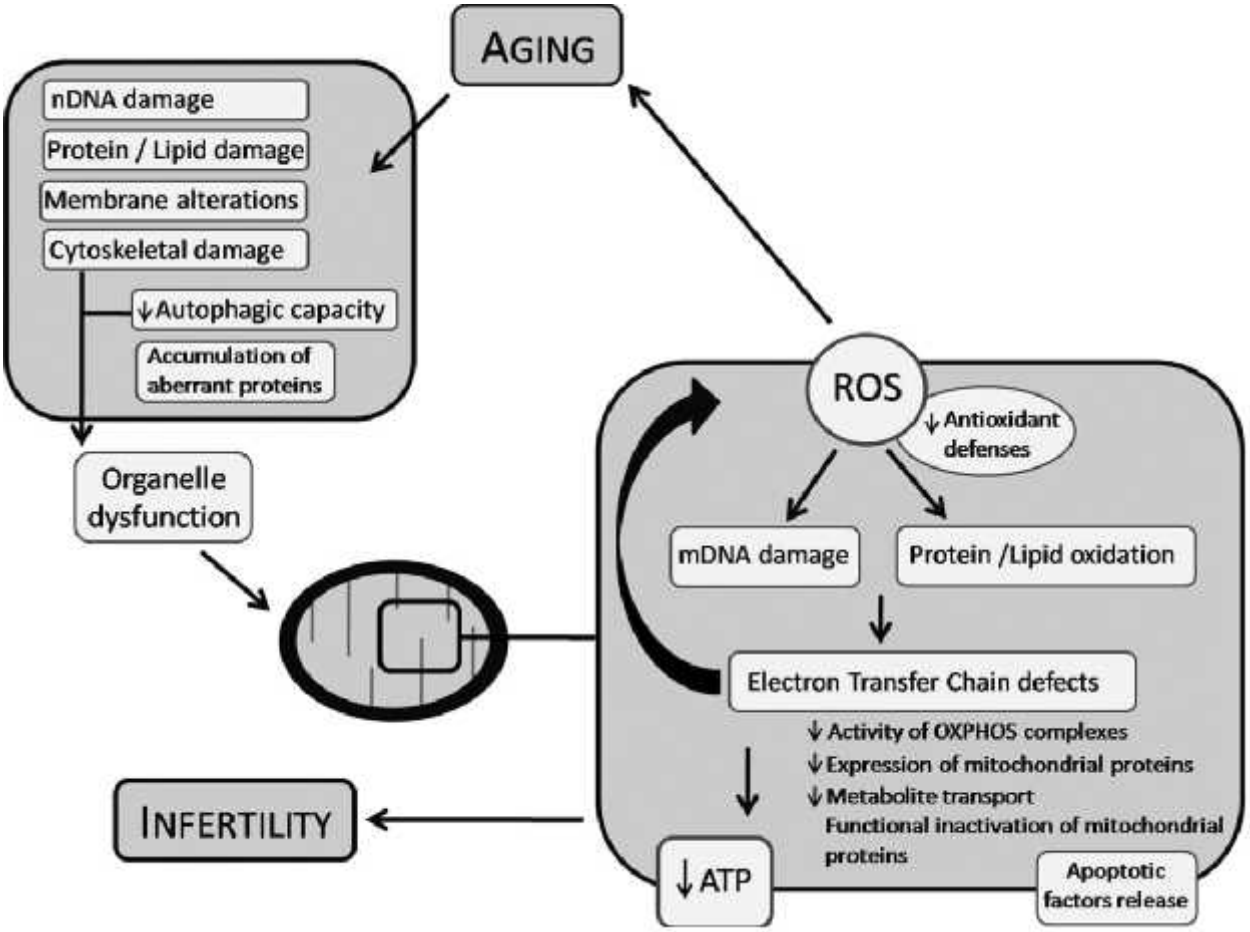

Figure 3. Effects of aging and the mitochondrial connection. Increased production of ROS leads to mDNA damage, and consequently to a general decline in mitochondrial function that, in a vicious cycle-like effect, results in more oxidative stress. Mitochondrial function decline and oxidative stress response in aging seems to have implications for fertility, mainly due to the resultant energy crisis. An increase in proton leak, mediated by UCP2, may represent an adaptive strategy to overcome the increased production of ROS (see text). Therefore mitochondria seem be a common link between aging and the age-related decline in reproductive function.

phosphorylative function correlate with the rat reproductive cycle, exhibiting a peak of functionality in fully adult animals and being depressed in older animals (72). Similar effects were also reported in the cat (73). Although a declining mitochondrial function in older animals was observed, there was also evidence for the triggering of protective mechanisms, namely the increase in mitochondrial UCP2 content and function, which can promote proton leak, and attenuate ROS levels by causing a controlled decrease in mitochondrial membrane potential. Thus, age-induced changes in reproductive function may be caused by testicular mitochondrial dysfunction that may lead to a decline in ATP synthesis and a consequent energy crisis affecting the maintenance of testicular homeostasis (72) (Figure 3).

Moreover, mitochondria from Leydig cells are also affected by age, a phenomenon that might be related with the decrease in testosterone production. The number of Leydig cell mitochondria seems to be reduced in the aging male and StAR protein levels, as well as expression and activity of steroidogenic enzymes, are also lower (149-150) (Figure 2). Using a long-term suppression of steroidogenesis model, Chen and Zirkin observed the abolishment of age-associated alterations indicating that steroidogenic activity and associated ROS production are probably the main cause for these effects (151). These results match previous work that, in a similar model, observed an increase in lipid peroxidation after the resumption of steroidogenesis, with the steps regulated by P450 enzymes as the most likely sites of free radical generation (152). Subsequently Chen and coworkers observed age-related changes in the production of ROS by Leydig cell mitochondria, with those of older Leydig cells producing significantly greater levels of ROS than those from younger ones. Furthermore, absolute mitochondrial volume was also reduced in old cells. The results are consistent with the proposal that mitochondrially-derived ROS may play a role in the irreversible decline in the ability of older Leydig cells to produce testosterone (153).

In accordance, Luo and collaborators, observed an age-related decrease in the antioxidant machinery of Leydig cells, a situation that can contribute to the previously observed increased oxidative damage (154). In a different study, Lacombe and colleagues obtained concordant results. Using KO mice for the Pituitary adenylate cyclase-activating peptide (PACAP) they observed that testicular aging was delayed probably due to a decline in ROS production in KO mice (155).

That mitochondrial dysfunction might be a link between aging and fertility reduction has also been validated by data obtained with genetically engineering mice. Unfortunately, genetic manipulations to study the involvement of mitochondria in aging and reproduction are challenging, as mutations in genes encoding mitochondrial proteins often result in embryonic lethality. At any rate some interesting clues have been published so far. For instance, homozygous knock-in mice expressing a proofreading form of DNA polymerase gamma (POLG; the sole DNA polymerase in mitochondria), and which develop a mutated mtDNA phenotype, with increased mtDNA mutations and deletions, progressively acquire premature aging traits, including reduced fertility (156). From 12 weeks of age onwards, testes of mtDNA-mutator males were smaller than wild-type testes, and the epididymal sperm content was also reduced. The authors also observed severe testicular tubular degeneration, with complete absence of sperm in 40-week-old mtDNA-mutator mice. Likewise, the testes of testis-specific cytochrome c KO mice underwent early atrophy similar to what occurs in aging, as a consequence of reduced OXPHOS (157). Furthermore, male mice with a mutation in the inner mitochondrial membrane peptidase 2-like (Immp2l), affecting the signal peptide sequence processing of the mitochondrial proteins cytochrome c1 and glycerol phosphate dehydrogenase 2, and resulting in mitochondrial hyperpolarization and high superoxide ion generation, were severely subfertile due to erectile dysfunction (158). The seminiferous tubules of 7 months old mutant mice (but not younger ones) showed marked disorganization, vacuolization and reduced number of germ cells, and also a higher rate of germ cell apoptosis.

Lastly, the possible role of mitochondria in age-dependent decrease of male fertility can also be substantiated by sperm parameters. Actually, it has recently been demonstrated that sperm mitochondria do produce ROS, which make a significant contribution to oxidative stress in the male gamete (159), and might play a key role in age-related reproductive pathophysiology (119). Moreover, and although the ability of sperm to enter the apoptotic pathway has been doubted, recent data provided evidence that mature human sperm can be induced to undergo a limited form of apoptosis characterized by mitochondrial ROS generation, phosphatydylserine externalization, caspase activation, motility loss, cytoplasmic vacuole formation and oxidative DNA damage (160). The authors suggested that the purpose of this apoptotic response may be to facilitate the silent phagocytosis of senescent moribund sperm within the female tract, and to prevent oxidatively damaged sperm from participating in fertilization. However, such a natural selection can be bypassed by the increased use of intracytoplasmic sperm injection (ICSI), with the concomitant risk of transmitting genetic errors to the new generations. Such a risk may be higher for older fathers, given that ejaculates may contain a bigger subpopulation of apoptotic sperm in this case. Better approaches should be designed in order to deplete sperm from such a subpopulation prior to any ART. Moreover, the likelihood that sperm from older fathers may contain epigenetic defects, which can potentially be transmitted to the embryo, should also be investigated in order to better understand the true magnitude of the risk of fatherhood at later ages.

In conclusion, mitochondrial dysfunction is negatively correlated with, and may affect, several reproductive parameters. Further studies will pinpoint the exact mechanisms governing age-dependent changes in male fertility.

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Send correspondence to: Joao Ramalho-Santos, Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal, Tel: 351239-855 760, Fax: + 351(239)-855 789, E-mail: jramalho@ci.uc.pt