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Chapter 1

SPERMATOGENESIS AT THE EXTREME: OXIDATIVE STRESS AS A CONVERGING MECHANISM OF TESTICULAR DAMAGE DUE TO PATHOLOGICAL AND ENVIRONMENTAL EXPOSURE

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ABSTRACT

Mammalian spermatogenesis is a complex physiological process that takes place inside seminiferous tubules in the testis. This process involves a fine balance between cell proliferation, differentiation and apoptosis, ending up with a motile spermatozoon. In most mammals the testes are kept 4-5 °C below body core temperature in the scrotum and the spermatogenic process proceeds with a blood and oxygen supply that appears to be fairly independent of changes in other vascular beds in the body. Despite this well-controlled environment, special circumstances (i.e. genetics), lifestyle, occupational work, pathologies (e.g. diabetes, cryptorchidism and varicocele) and environmental exposure to low oxygen (hypoxia) can result in a decrease of blood flow, nutrients and oxygen supply along with increased temperature. All of these conditions, besides those that cause some pathological conditions, may lead to subfertility or infertility. However, they also represent unique conditions that help us to address some crucial questions about

the cell biology and physiology of spermatogenesis: what advantage is there for the spermatogenic cell processes mentioned above taking place under mild hypothermia? What are the molecular and physiological processes that make spermatocytes especially vulnerable to high temperatures? What mechanisms of adaptation take place in the seminiferous tubules cells under hypoxia? What mechanisms lead to a decreased sperm output from spermatogenesis under hypoxia? Do these conditions have similar physiological and molecular pathways? In this chapter, we will review the available literature on human and animal models concerning "extreme" conditions for spermatogenesis, such as: heat stress, hypoxia and pathological conditions like varicocele, cryptorchidism and diabetes. Our analyses suggest that germ cell apoptosis, oxidative stress and DNA damage are common features in all of these "extreme" conditions. Furthermore, oxidative damage seems to be present in all of these conditions during the initiation step of cellular damage. This oxidative stress in the testicle is critical when it poses an imminent risk to the viability and quality of the reproductive cells of animals and humans subjected to extreme conditions. Thus, about 25% of infertile patients are diagnosed with elevated levels of reactive oxygen species (ROS) in semen samples. It is well documented that oxidative damage and elevated ROS levels in germ cells lead to apoptosis, a likely cause of hypospermatogenesis and low sperm production in these patients.

Keywords: Testis, apoptosis, oxidative damage, hypoxia, infertility, germ cell

INTRODUCTION

Oxidative and reductive chemical processes are inherent to life on earth and to the processes that use oxygen as a final electron acceptor in one-electron transference, inevitably producing reactive oxygen species (ROS) that can also lead to the formation of reactive nitrogen species (RNS), which can modify biomolecules and affect lipids, proteins and oligonucleotides. Undoubtedly, these same chemical modifications by ROS/RNS have been evolutionarily selected for as physiologically meaningful modifications and are used as signalling processes in cells. Furthermore, counteractive antioxidant mechanisms are widespread throughout biological systems and provide the necessary balance for oxidative processes to be tamed and regulated at useful levels in cells. However, many conditions that increase ROS/RNS or decrease the antioxidant capacity of cells and tissues are at the basis of many pathological situations characterized by programmed (apoptosis) or necrotic cell death and, in some cases, by carcinogenic cell transformation.

In this chapter we review some of the "extreme conditions" that the testis can be subjected to, which appear to have oxidative stress as a common denominator, leading to cell apoptosis, necrosis or carcinogenic cell transformation of testicular cells.

HUMAN PATHOLOGIES ASSOCIATED WITH TESTICULAR OXIDATIVE STRESS

The causes of male infertility are extremely varied. Some infertile men have a complete absence of spermatozoa in the ejaculate that could be due to an obstruction of the deferent ducts (obstructive azoospermia) or to a failure in the spermatogenic process inside the testis

(non-obstructive azoospermia), including hypospermatogenesis, maturation arrest or Sertoli cell-only syndrome. Other infertile men are able to produce spermatozoa but they have severe morphological alterations (teratozoospermia), they are completely immotile (asthenozoospermia) and/or they are produced in very low quantities (oligozoospermia).

The aetiology of male infertility due to non-obstructive conditions is as varied as the the possible diagnoses from semen analysis. Some of the factors recognized so far as being associated with non-obstructive infertility are microdeletions in the Y-chromosome (Sadeghi-Nejad and Farrokhi, 2007), failures in the hypothalamus-hypophysis-gonad axis (Sokol, 2009), exposure to certain pesticides used in agriculture (Jurewicz et al., 2009) and pathological conditions such as cryptorchidism (Chung and Brock, 2011) and varicocele (Baazeem et al., 2011). The implications of certain lifestyle factors such as laptop, Wi-Fi and cell phone usage on male infertility are still under debate (Sheynkin et al., 2005; Agarwal et al., 2008; De Luliis et al., 2009; Falzone et al., 2010, Avendaño et al, 2011).

The mechanisms by which different factors generate male infertility status are diverse. However, it has been postulated that an intratesticular and/or seminal increase in ROS levels is a common characteristic in most cases. In this first part, we will explore the hypothesis that a decrease in the testicular oxygen supply and/or an increase in gonadal temperature are causes of male infertility.

Numerous daily activities are associated with an increase in scrotal temperature, such as laptop use (Sheynkin et al., 2005) and heated car seats (Jung et al., 2008), which could negatively affect seminal parameters due to the extremely high sensitivity of germ cells to heat stress (Figure 1); however, the correct regulation of scrotal temperature and the cyclic process of spermatogenesis in healthy men prevents permanent germ cell damage caused by testicular hyperthermia (Momen et al., 2010).

On the other hand, it has been postulated that failure to maintain normal scrotal temperature and prolonged gonadal exposure to high temperatures could be part of the aetiology of male infertility, which is supported by the observation that subfertile men have a significantly higher intrascrotal temperature compared to normozoospermic men (Mieusset et al., 1987; Zorgniotti and Sealfon, 1988).

Regarding the effects of a decrease in the testicular oxygen supply on male infertility, chronic hypoxia induces a state of reversible oligozoospermia in healthy men (Verratti et al., 2008); however, if the hypoxia is permanent, it generates ischaemic damage (Gat et al., 2006). Moreover, some investigators have suggested that long-standing intratesticular hypoxia could be part of the aetiology of certain severe primary spermatogenic failures, such as maturation arrest and Sertoli cell-only syndrome (Gat et al., 2010a; Gat et al., 2010b).

The mechanism by which a permanent decrease in oxygen supply and/or increase in gonadal temperature generate germ cell impairment is most likely mediated by an increase in intratesticular or seminal ROS (Figure 1). Although these molecules have a physiological role in the spermatogenic process, a pathological increase in their concentrations would negatively affect the survival and differentiation of germ cells (Amaral et al., 2008). Indirect evidence indicates that intratesticular ROS levels in men with severe male infertility conditions, such as Sertoli cell-only syndrome and maturation arrest, are significantly increased compared to the levels detected in men with conserved spermatogenesis (Koksal et al., 2003) and that ex-cryptorchidic men with a diagnosis of oligozoospermia have greater levels of sperm DNA damage and ROS levels in their semen compared to normozoospermic healthy controls (Smith et al., 2007).

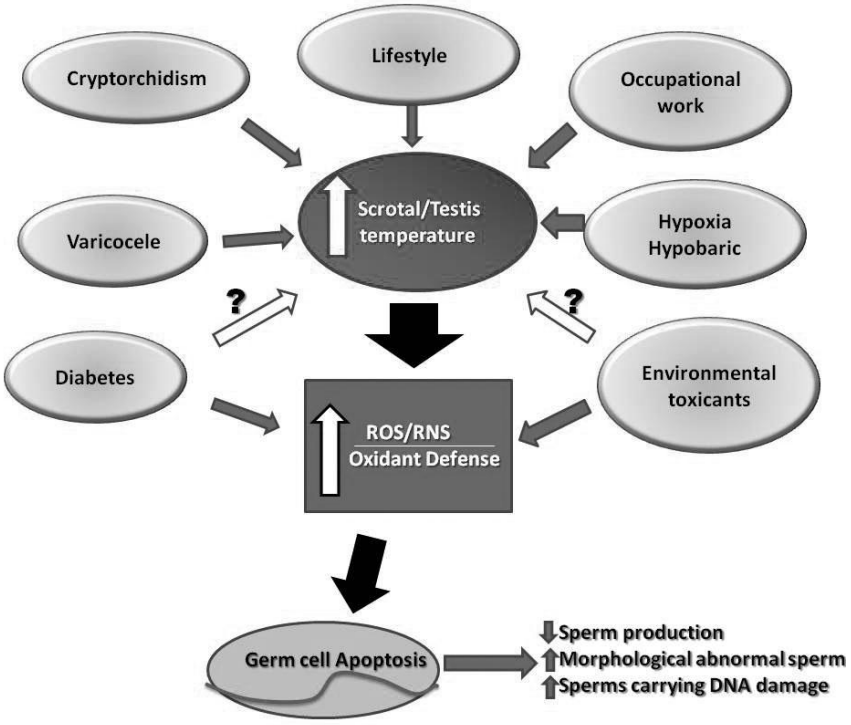

Figure 1. Pathological and environmental factors affecting spermatogenesis. Many male tract pathologies such as cryptorchidism and varicocele are strongly associated with an increase in temperature (1-2 °C) in the testes. Other pathologies, such as diabetes, can also induce an increase in testes temperature. Interestingly, other conditions such as lifestyle, occupational work and hypobaric hypoxia lead to an increase in temperature in the testes and oxidative stress, similar to varicocele and cryptorchidism. The white arrows for the diabetes and environmental toxicants indicate that no experimental data showed a role of these conditions in testes heat stress. Heat stress leads to an imbalance between the production of reactive oxygen species (ROS/RNS) and the antioxidant system (enzymatic and non-enzymatic). This oxidative stress could lead to many defects in testicular physiology; among them, an increase in germ cell apoptosis seems to be the main factor for the reduction in the number of sperm produced. In addition, oxidative stress could account for the increased levels of DNA damage and abnormal spermatozoa.

An important pathology associated with male subfertility and an increase in intratesticular and seminal ROS levels is varicocele. This pathology consists of the thickening and dilation of the pampiniform plexus above the testis, but its aetiology is still unknown. A diagnosis of varicocele is made in 35% of men with primary infertility and in approximately 80% of men with secondary infertility (Belloli et al., 1993). It is postulated that varicocele causes germ cell apoptosis by increasing the hydrostatic pressure in the testicular venous system through a malfunctioning of the testicular valves, leading to an intratesticular hypoxic environment (Gat et al., 2006). However, an alternative hypothesis is that high temperature rather than intratesticular hypoxia is the main source of oxidative stress in patients with varicocele, which is supported by three lines of evidence: 1) patients with varicocele have a

higher scrotal temperature and shorter recovery period after cold stress (Merla et al., 2002); 2) varicocelectomy improves semen parameters, probably by reducing testicular temperature and ROS levels and increasing antioxidant activity in the seminal plasma (Wright et al., 1997; Mostafa et al., 2001); 3) increased scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis (Shiraishi et al., 2010).

It is generally accepted that testicular and seminal ROS levels are important in the deleterious effects of varicocele on male fertility. Thus, ROS levels in semen samples from men with varicocele are significantly higher compared to those of fertile control men (Smith et al., 2006), and they are directly related to the degree of varicocele (Mostafa et al., 2011). In this regard, the damage caused by varicocele becomes worse as the time between its first appearance and varicocelectomy surgery increases (Zorba et al., 2009), indicating that the testes have intrinsic mechanisms for avoiding permanent damage under certain conditions, but that damage is inevitable when the hypoxia (or elevated temperature) is sustained over time.

Finally, in the last few years it has been proposed that some daily activities and somatic pathologies (not strictly related to fertility) such as the use of cell phone and diabetes negatively affect male infertility through an increase in intratesticular and/or seminal ROS levels via mechanisms that do not necessarily involve heat stress or hypoxia. Regarding the use of cell phones, this is one of the most conflict-ridden points. Recent studies showed that the excessive use of this device may impair some seminal parameters such as sperm count, motility, viability and normal morphology, a phenomenon that is dependent on the duration of daily exposure to cell phones and independent of initial semen quality (Agarwal et al., 2008; Gutschi et al., 2011). The mechanisms involved in this effect consist of an increase in ROS production with subsequent DNA damage in spermatozoa (De Iuliis et al., 2009), which is caused more by the radiofrequency of electromagnetic radiation than by the heat emitted from these devices (Mailankot et al., 2009). These results suggest that excessive cell phone use could be regarded as an "extreme condition" and a deleterious factor for spermatogenesis.

Obesity and diabetes have become two of the greatest threats to human health during the last century. Diabetes mellitus is a metabolic disorder characterized by insulin resistance and subsequent hyperglycaemia (Amaral et al., 2008; Cabler et al., 2010; Du Plessis et al., 2010). Diabetes I is an autoimmune disease caused by the inadequate secretion of insulin that appears in early childhood or puberty and involves the selective loss of beta-cells in the pancreas (Boitard, 2002). Diabetes II is produced by deficiencies in insulin signalling levels and is the most prevalent type of diabetes (Shulman, 2000). Obesity is considered a factor in the development of diabetes II through a mechanism associated with insulin resistance (Shulman, 2000). Diabetes and obesity are part of the cluster of metabolic diseases that, among other dysfunctions, seem to impair reproductive life (Cabler et al., 2010; Du Plessis et al., 2010). Growing evidence shows a strong link between obesity/diabetes and decreased libido, impotence and impairments in testicular functioning, which are associated with sustained hyperglycaemia (Cameron et al., 1990; Cabler et al., 2010). In this way, sperm parameters in semen such as concentration, motility and morphology are altered in obese men (Jensen et al., 2004; Hammoud et al., 2006; Hakonsen et al., 2011). Research performed on 835 diabetic men native to Qatar indicated that 35% were infertile, exhibiting primary or secondary infertility (Bener et al., 2009). Sperm parameters of diabetic men, like motility and density, are poorer than in healthy men (Sexton and Jarow, 1997; La Vignera et al., 2011). In addition, spermatozoa of diabetic men have greater levels of DNA damage and lipid

peroxidation than that of normal healthy men (Kasturi et al., 2008). However, classic semen parameters do not always show subtle, functional damage in nuclear and mitochondrial sperm DNA (Agbaje et al., 2007; Agbaje et al., 2008). Furthermore, a decrease in seminiferous tubule diameter, along with germ cell exfoliation, alterations in the connection of Sertoli cells with spermatids and vacuolization of Leydig cells and Sertoli cells were observed in a study that involved ten diabetic patients (six of whom had diabetes I) (Cameron et al., 1985).

The establishment and progression of spermatogenesis depends on intratesticular concentrations of testosterone, a hormone produced by Leydig cells inside testes that works through receptors located in Sertoli cells. Lower levels of serum testosterone have been found in patients with diabetes type I compared to healthy individuals, and hyperglycaemia reduces the response of the pituitary gland when it is stimulated by gonadotropins (Distiller et al., 1975; Garcia-Diez et al., 1991). It has been reported that the conversion of testosterone to oestrogen is increased in obese individuals compared to non-obese subjects, which could be a cause of hypogonadism, presumably by the deleterious role of oestrogen in germ cell survival (Cabler et al., 2010; Du Plessis et al., 2010).

Another important element in obese men is the accumulation of adipose tissue in the suprapubic and scrotal region (Figure 2). Adipose tissue in this area can increase the temperature of the testes, which induces oxidative damage in germ cells (Kasturi et al., 2008). At this point, it is important to note that male reproductive dysfunctions in diabetes do not strictly require the simultaneous occurrence of obesity.

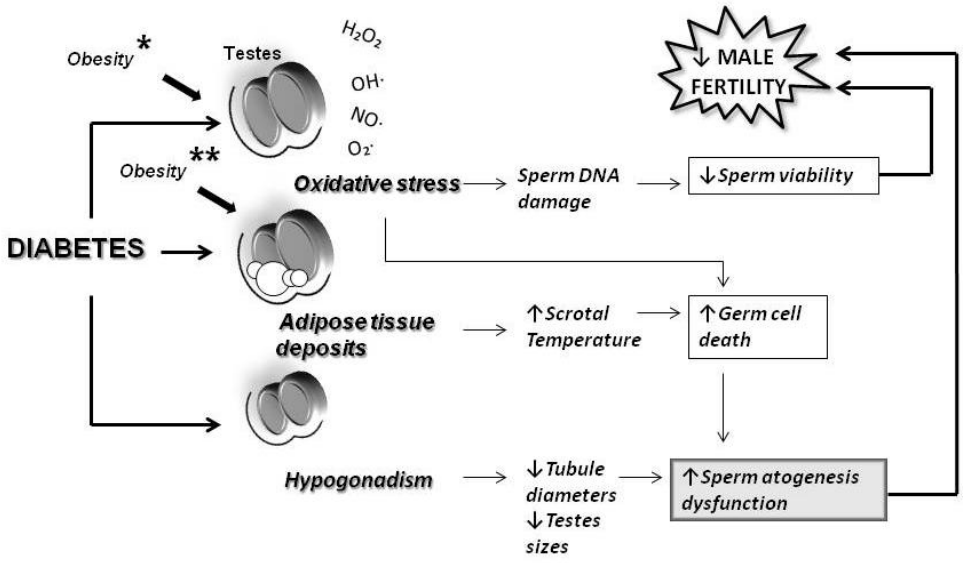

Figure 2. Mechanisms that trigger spermatogenesis dysfunctioning and the reduction of male fertility in diabetes. There is experimental evidence to show that oxidative stress is a factor that increases germ cell death, causing a dysfunction in spermatogenesis. In addition, oxidative stress damages sperm DNA, decreasing sperm viability. Hypogonadism is also an impairment that is found in diabetic subjects, which also causes sperm alterations. (*) Obesity is a factor that could contribute to spermatogenesis dysfunctioning in diabetes by increasing oxidative stress. (**) When diabetic men are obese, adipose tissue deposits build up around the testes and seminal cord, increasing the temperature.

The currently available evidence described in this section indicates that intratesticular hypoxia, increased temperature (heat stress), hyperglycaemia and possibly electromagnetic radiation exposure through the use of cell phones could lead to oxidative stress and impair spermatogenesis with a consequential increase in germ cell apoptosis and DNA damage in spermatozoa. In the next sections we will review experimental evidence using animal models in order to clarify the real contributions of hypoxia, heat stress and hyperglycaemia to the impairment of spermatogenesis through oxidative stress.

ANIMAL MODELS ASSOCIATED WITH HYPOXIA

Hypoxia is a stress factor that generates a series of physiological changes in order to compensate for low oxygen tension. In studies of the effects of hypoxia in the testes, the epididymis and spermatozoa are of great relevance in the area of animal reproduction in light of current climatic changes and the increasing exposure of aquatic and terrestrial animals to extreme conditions of low oxygen tension.

Aquatic invertebrates are constantly being exposed to environmental oxygen concentration and temperature fluctuations (Gorr et al., 2010); as a consequence, reports of hypoxia have increased and are a cause of concern. In recent years, investigations that mainly focused on adult fish have shown that aquatic chronic hypoxia acts as an endocrine disruptor, leading to a decrease in gonadal growth, gametogenesis and a reduction in fertility of the Atlantic sea bass. In addition, a delay in spawning has been observed in Gulf killifish (*Fundulus grandis*), whereas in fish embryos hypoxia leads to defects in the migration of primordial germ cells to the genital ridge (Lo et al., 2011). Endocrine and reproductive dysfunctions have also been observed in fresh water species such as carp (Thomas et al., 2007). Another type of adaptation has been observed in the planktonic crustacean *Daphnia magna*, as well as in *Drosophila melanogaster*, through the regulation of different globin genes driven by the hypoxia-inducible factor (HIF), mainly influencing angiogenesis in the tracheal system (Ratcliffe et al., 1998; Gorr et al., 2010). Tracheas in insects, as well as lungs and blood vessels in mammals, are the best examples of successful adaptations that animals have shown over the course of evolution for taking up and delivering O_2 (Centanin et al., 2010).

Exposure to low levels of environmental oxygen is of great concern in domestic animals, which are not genetically adapted to altitude (GAA), unlike llamas or alpacas. The absence of chronic mountain sickness (CMS) in GAA is linked to various adaptations such as the absence or small erythrocytosis because haemoglobin has a great affinity for oxygen (Leon-Velarde and Mejia, 2008). GAA animals do not exhibit a vasoconstrictor response to hypoxia, which avoids the sustained elevation of pulmonary arterial pressure and the subsequent right ventricular hypertrophy found in humans and domestic animals at high altitudes (Leon-Velarde and Mejia, 2008). Previous studies on male rat models indicated that hypoxia reduces fertility when animals do not adapt to this condition, reducing sperm motility in semen and sperm count (Cikutovic et al., 2009; Farias et al., 2010). A reduced sperm count could be associated with an increase in apoptosis promoted by this hypoxic condition (Liao et al., 2010). The same results were observed in male rhesus monkeys (Saxena, 1995). Morphological studies have revealed that hypoxia causes degeneration of the germinal

epithelium, folding of the basement membrane, degeneration and detachment of germ cells, lipid droplet sedimentation in Sertoli cells, and an increase in lipoperoxidation (Farias et al., 2005a; Liao et al., 2010). Local changes in testicles have also been observed including neovascularization, an increase in scrotal temperature, a decrease in testicular mass and an increase in interstitial space (Farias et al., 2005b). Interestingly, some of these changes have been observed in humans with varicocele (see above), suggesting that they may have a similar aetiology.

Studies on animal models indicate that varicocele induces testicular hypoxia (Wang et al., 2010). Varicocele is a dilation of the veins of the spermatic cord, determined by dilation of the spermatic vein and stasis (Fazlioglu et al., 2008). An increase in the hypoxia-inducible factor 1 alpha (HIF-1α) levels was previously observed in a rat model, which is an extrinsic marker of hypoxia, generating an increase in vascular endothelial growth factor (VEGF) and followed by angiogenesis, in addition to promoting germ cell apoptosis (Wang et al., 2010). For these reasons it is said that testicular hypoxia can affect fertility, when bilateral varicoceles are produced (Gat et al., 2006; Tekcan et al., 2011). Changes in testicular tissue have been described in both humans and animal models of varicocele, particularly in rats, including immature, amorphous and small cells (Fazlioglu et al., 2008), producing smaller testicles with a decrease in Leydig cell functioning and a low total sperm count, but with no abnormalities in the motility or morphology of the spermatozoa. The reduced sperm count is significant and directly proportional to the grade of varicocele.

As described above, environmental hypoxia affects mainly two testicular functions: first, spermatogenesis is impaired with spermatogenic cell apoptosis and, second, testosterone production is also affected by long-term hypoxia (Farias et al., 2008). Only a few studies have addressed the effects of hypoxia in isolated testicular cells. In a Leydig cell-like cell line (TM3), Lysiak et al. (2009) reported that hypoxia was able to increase the concentrations of HIF-1α and the 3β-OH steroid dehydrogenase, an enzyme in the androgen biosynthetic pathway, strongly suggesting that a direct effect of hypoxia on Leydig cell function could explain the changes in testosterone observed in animal models of hypoxia. Mitochondrial measurements in isolated spermatids from hypoxia-treated rats strongly suggested that these cells undergo lipid peroxidation. Furthermore, our group recently performed experiments to show HIF-1α responses of isolated seminiferous tubules or spermatogenic cells under chemical inhibition of prolyl hydroxylases (PDH), the enzymes that sense oxygen and control HIF-1α levels in cells. Our results strongly suggest that the prepubertal seminiferous tubules and spermatogenic cells respond with a high sensitivity to chemical hypoxia (Alliende et al., unpublished results). Regarding these aforementioned experiments, it is worth noting that inducing chemical anoxia by inhibiting mitochondrial oxidative phosphorylation is not the same as simulating hypoxia by inhibiting PDHs. The former approach is closer to a model of cellular anoxia and the latter method is closer to a model of cell hypoxia.

ANIMAL MODELS ASSOCIATED WITH HIGH TEMPERATURE IN THE TESTES

The most frequently used experimental protocol for inducing hyperthermia in the testes is to immerse the lower third of the animal (usually a mouse or rat) in a warm bath (normally at 43 °C) for a period of time, ranging from 15 to 30 min (to cause heat stress). It is understood that this experimental protocol is akin to the environmental conditions experienced in normal human life, such as a shower in warm water, the wearing of tight underwear, the sitting position in the workplace, and pathological conditions such as increased testicular fat or increased blood flow.

Heat stress induces a general change in the transcriptome of mice and human testes, and a total of 67 transcripts were found to be heat regulated in C57BL/6 mice (Li et al., 2009). In another study using ICR mice with a heat stress protocol of 35 °C for 24 h, it was shown that 225 genes were differentially expressed (Cammack et al., 2009). On the other hand, in humans, it was shown that 31 and 36 known proteins are differentially expressed at 2 and 9 weeks after heat treatment, respectively (Zhu et al., 2010).

Under normal conditions the highest rate of germ cell apoptosis is observed in early zygotene and ending pachytene spermatocytes (stages I and XII for rats and mice). The evaluation of germ cell apoptosis 1-2 days after heat stress showed a significant increase in apoptosis, mainly in the early (I–IV) and late (XII–XIV) stages, where pachytenes, dividing spermatocytes and early spermatids were the most frequent cell types observed undergoing apoptosis (Lue et al., 1999). In addition, a reduction in Cdc2 levels was observed in mice testes after heat stress, and in rabbit testes after experimental unilateral cryptorchidism (Kong et al., 2000; Zhang et al., 2008). Eventually, spermatogenesis recovers to levels similar to non-treated animals because spermatogonia are relatively heat resistant, with the exception of B-type spermatogonia of rams (Waites and Setchell, 1990; Lue et al., 1999) and bulls. Interestingly, isolated haploid germ cells but not somatic cells undergo apoptosis at 37°C under the same culture conditions, indicating that heat stress only activates the apoptotic pathway in germ cells (Yin et al., 1998).

The mechanism by which heat stress induces apoptosis in germ cells has yet to be defined. In rats and monkeys heat stress induces translocation of the pro-apoptotic protein BAX from the cytoplasm to the mitochondria, where it helps to release cytochrome c (Lue et al., 2002; Hikim et al., 2003; Matsuki et al., 2003; Vera et al., 2004; Jia et al., 2009). Caspase activation is a hallmark of apoptosis; during heat stress caspase-9 and 3 become active, and their pharmacological inhibition prevents germ cell death (67%), suggesting that caspases are directly linked to germ cell death after heat stress (Hikim et al., 2003; Matsuki et al., 2003; Vera et al., 2005).

The generation of ROS in the initiation of apoptosis is of paramount importance in germ cell apoptosis and DNA damage (Makker et al., 2009). Reactive oxygen species are molecules that have at least one unpaired electron and they are highly unstable and reactive in the presence of lipids, amino acids and nucleic acids (Franco et al., 2008; Hirano, 2008; Tuteja et al., 2009). At physiological levels, ROS are essential for normal reproductive functioning, acting as metabolic intermediates and regulating vascular tone, gene expression and sperm capacitation (Sikka, 2001; Franco et al., 2008; Hirano, 2008; Makker et al., 2009; Tuteja et al., 2009). Heat stress induces oxidative stress, triggering cell survival and apoptosis

depending upon the cell type and the extent of the insult. Many studies on heat stress focused on ROS-generating enzymes that produce ROS as by-products of their enzymatic activity. Xanthine oxidase (XO) catalyses the conversion of hypoxanthine and xanthine to uric acid, producing hydrogen peroxide as a by-product, and XO inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism (Kumagai et al., 2002). However, there is a lack of information about whether or not other ROS-generating enzymes such as cyclooxygenase (COX), lipoxygenase (LOX) and NADPH oxidase (NOX) are activated after testicular heat stress. In addition, it has also been shown that the major source of non-enzymatic ROS generation in aerobic cells comes from mitochondrial sources, specifically complex I and complex III. Under normal conditions, 1-2% of all oxygen consumed during respiration undergoes a single electron reduction by leakage of electrons from quinone pools to form the superoxide radical (Raha and Robinson, 2000; Raha et al., 2002; Ott et al., 2007). Then, mitochondrial superoxide dismutase (SOD) converts superoxide into hydrogen peroxide, which is then converted to water and oxygen by catalase and glutathione peroxidase (GPx). Under stress conditions the amount of electrons that leak out is enhanced, leading to an increase in, and the accumulation of, superoxide in mitochondria. It has been shown in both fungi and skin cells that mitochondrial dysfunction and XO are the major sources of ROS production (Davidson and Schiestl, 2001; Shin et al., 2008). The enzyme GPx produces water from H_2O_2 using glutathione (GSH) as the electron donor (Meister and Anderson, 1983). The levels of GSH are maintained by glutathione reductase (GR), which recycles oxidized glutathione (GSSG) to GSH using NADPH as the electron donor. Glutathione reductase activity is modulated by ROS levels although ascorbic acid helps to prevent this effect (El-Missiry, 1999). Nitric oxide (NO) is synthesized intracellularly through the action of a family of nitric oxide synthetase (NOS) enzymes. These NOS enzymes catalyse the NADPH- and O_2-dependent oxidation of L-arginine to L-citrulline, producing NO (Hill et al., 2010). This molecule is a free radical and is chemically more stable and less reactive than other ROS such as the superoxide anion or hydrogen peroxide (Pacher et al., 2007). In monkey testes, endothelial nitric oxide synthetase (eNOS) and inducible NOS (iNOS) were found to be expressed in Sertoli and germ cells. No obvious alterations in eNOS levels were detected after heat stress, but the levels of iNOS increased 3 days after treatment compared to the controls. Immunocytochemistry showed a robust increase in iNOS expression in germ cells (Guo et al., 2009). Thus, heat stress induces NO production and it might contribute to oxidative damage in germ cells. However, the molecular targets that are modified by NO production and the consequences of this in testis physiology are unknown.

Under different conditions the production of ROS exceeds the antioxidant capacity, resulting in oxidative stress, which elicits degenerative responses such as DNA damage, necrosis, and apoptosis. Heat stress was found to increase apoptosis and ROS levels in isolated germ cells (Ikeda et al., 1999). Interestingly, oxidative damage, along with increased apoptosis, is also a hallmark of other pathologies involved in male infertility, such as varicocele and testicular torsion (Turner et al., 2004; Turner et al., 2005; Shiraishi and Naito, 2007; Shiraishi et al., 2010). Heat stress causes the upregulation of haeme oxygenase 1 (Hmox1) and the antioxidant enzymes glutathione peroxidase 1, glutathione S-transferase alpha and SOD1, consistent with a robust oxidative stress response. In agreement with the hypothesis that these enzymes participate in the antioxidant response, germ cells isolated from SOD1-knockout mice were found to be more vulnerable to apoptosis induced by heat stress than wild-type mice, suggesting a protective role of this enzyme in the oxidative stress

insult induced by heat stress (Ishii et al., 2005). However, other studies reported a downregulation of mRNAs encoding for glutathione reductase 1, glutathione S-transferase alpha 2, manganese superoxide dismutase 2 (SOD2) and extracellular superoxide dismutase Cu-Zn (SOD3) (Rockett et al., 2001). Thus, it is seems that heat stress induces the upregulation of specific isoforms of stress response enzymes (SOD1 but not SOD2 or SOD3), indicating the activation of a specific genetic response programme. Peroxiredoxin-4 (Prx4) belongs to a new family of antioxidant proteins and it plays a role in regulating NF-κB (nuclear factor κB). Male Prx4-knockout mice are fertile, except for testicular atrophy and an increase in germ cell apoptosis (Iuchi et al., 2009). Interestingly, Prx-4-knockout germ cells show increased oxidation of lipids and protein thiols, and male mice show an increase in apoptosis (TUNEL-positive cells) after heat stress, suggesting that Prx4 is important in the oxidative stress response in germ cells.

Different intracellular pathways are activated downstream ROS production, among which the ASK1/p38MAPK pathway has been shown to be important in germ cell apoptosis after heat stress (Ueda et al., 2002; Matsuzawa and Ichijo, 2008). Reactive oxygen species are able to oxidize thioredoxine (Trx)-reactive thiol groups and to mediate the activation of apoptosis signal-regulating kinase 1 (ASK1)/MKKK5, a ubiquitously expressed enzyme that activates JNK and p38 MAP kinase pathways (Hsieh and Papaconstantinou, 2006; Matsuzawa and Ichijo, 2008; Hsieh et al., 2010). In rats and monkeys heat stress induces the sustained activation of both mitogen-activated protein kinase (MAPK) 1/3 and MAPK14 (p38MAPK) (Johnson et al., 2008; Jia et al., 2009; Lizama et al., 2009). Interestingly, the protection offered by caspase-2 or calpain inhibitors prevents MAPK14 (p38MAPK) activation, suggesting that caspase and calpain activation are upstream of MAPK activation (Johnson et al., 2008; Lizama et al., 2009).

Heat stress induces a gradual loss in sperm concentration and viability (Jannes et al., 1998; Setchell et al., 1998; Rockett et al., 2001; Paul et al., 2008b; Perez-Crespo et al., 2008). In monkeys, scrotal heat stress induces azoospermia or oligozoospermia 6 or 8 weeks after the first heat treatment (Lue et al., 2002), and heat stress in stallions induces increased susceptibility of spermatozoa DNA to denaturation in the absence of protamine changes (Love and Kenney, 1999). In rats, spermatocyte damage at the time of heat stress was found to result in lower sperm production 23-28 days after treatment; these sperm had reduced DNA integrity compared to the sperm of untreated rats, but spermatids at the time of heat stress showed the highest degree of DNA damage (Perez-Crespo et al., 2008). Moreover, after mating normal females to heat-exposed males, there was a reduction in the pregnancy rate and an increase in embryo reabsorption, and the surviving embryos experienced a reduction in weight during intrauterine development. *In vitro* fertilization with sperm recovered 16 h or 23 d after scrotal stress at 42°C produced embryos that experienced a block at the four-cell and blastocyst stage, suggesting a pre-implantational effect of heat stress in development and/or deficient egg activation (Paul et al., 2008a; Paul et al., 2008b). The reduction in the number and weight of embryos sired by heat-stressed males could be explained by the high levels of DNA damage, which promote abnormal embryos, yolk sacs, trophoblasts and placental development (Setchell, 1993; Setchell et al., 1998). Interestingly, a sex ratio distortion was found when spermatozoa present in the epididymis at the time of thermal stress produced embryos after mating (Perez-Crespo et al., 2008). Therefore, the available evidence clearly indicates that heat stress has a profound impact on spermatogenesis, with an increase in germ cell apoptosis and DNA damage in the spermatozoa produced by germ cells present at the

time of injury. Sperm DNA damage could be induced by a strong REDOX state imbalance. Despite the upregulation of many enzymes involved in the antioxidant response, some germ cells cannot cope with elevated ROS levels and they die by apoptosis (Figure 1). However, other groups of germ cells can overcome this oxidative stress and differentiate, but sperm produced this way bears high levels of DNA fragmentation and probably other, as yet unknown, harmful modifications, which could explain the developmental abnormalities observed in embryos after heat stress.

As described above, many studies have been performed on experimental hyperthermia, showing the triggering of apoptosis in pachytene spermatocytes (Nakai et al., 2000; Somwaru et al., 2004). In contrast, few studies of hyperthermia and its associated physiological and biochemical changes have been performed in isolated cells. The only study found in the literature using prepubertal monkey Sertoli cells (Chen et al., 2008) reported that adherent junction-associated proteins are downregulated by high temperatures (43 °C). On the contrary, vimentin expression is upregulated by high temperatures. These changes in cytoskeletal and junction proteins were thought to be associated with a marked decrease in androgen receptor (AR) expression after heat treatment, as was clearly demonstrated using selective Sertoli cell AR KO mice. Testosterone is essential for spermatogenesis through its actions on Sertoli cells. Thus, Sertoli cell AR downregulation by hyperthermia explains, at least in part, spermatogenic arrest in testis subjected to high temperatures. However, evidence showing the mechanisms of AR downregulation and spermatogenic cell apoptosis under conditions of high temperature is lacking. The interspecies extrapolation that we present herein is controversial, although it could certainly provide the grounds for new research experiments. Related to testosterone, and specifically being the source of testosterone in the testis, studies of the effects of high temperature on Leydig cell functioning and testosterone production are apparently absent in the literature. Regarding isolated germ cells, Herrera et al. (2000) showed that increasing the incubating temperature of rat pachytene spermatocytes and round spermatids to 37-40°C increased intracellular [Ca^{2+}] ([Ca^{2+}]i) to levels that modify signalling in these cells (e.g., Reyes et al., 2010). The intracellular pH in these cells was found to decrease with increasing temperature. These changes occurred within 1 minute of the increase in temperature and can be classified as early events in the response of these cells to heat stress. Interestingly, the same combination of changes in these cellular parameters (increase in [Ca^{2+}]i and decrease in intracellular pH) was associated with apoptotic cells in the testis (Lizama et al., 2007), strongly suggesting that high temperatures *per se* can set physiological conditions in spermatogenic cells that make them prone to other noxious or pro-apoptotic stimuli from Sertoli cells. Furthermore, we recently found that a high temperature (40°C) induced a rapid increase in reactive oxygen and/or nitrogen species in pachytene spermatocytes but not in round spermatids (Pino et al., unpublished results), providing the noxious stimuli that could differentially trigger cell death in spermatocytes.

Overall, experimental evidence strongly suggests that experimental or pathogenic heat stress (e.g. varicocele) induces oxidative stress, which in turn could induce apoptosis or create damage in germ cells in such a way they develop into spermatozoa carrying fragmented DNA.

Animal Models in Diabetes: A Tool for Studying Spermatogenic Damage

The animal models used to study spermatogenic damage and fertility disturbances in diabetes have several advantages:

- They allow studies on main organs and tissues that would be more difficult to study in humans, such as testes, the epididymis and the vesicula seminalis.
- Rats and mice easily develop diabetes after injections of streptozotocin.
- Rodents have a short life cycle: the time taken to reach puberty, undergo spermatogenesis onset and develop the collateral effects of streptozotocin-induced diabetes is short.
- They offer the possibility of repeating experimental assays under specific conditions in order to distinguish random from non-random events.

The typical models for studying spermatogenesis in diabetes, as well as other disorders linked to diabetes, are rats and mice; the former are used more frequently than the latter because they are less resistant to streptozotocin, the drug that induces diabetes via the selective destruction of beta-pancreatic islets (Ozturk et al., 1996). These diabetic animals (induced with a single dose of 40-80 mg/kg streptozotocin) exhibit a decrease in testis and epididymal weight (Shrilatha and Muralidhara, 2007). The histological alterations include a reduction in the diameter of or a smaller diameter of seminiferous tubules, atrophy, the absence of sperm, Sertoli cell vacuolization, fewer Leydig cells and germ cell desquamation (Ballester et al., 2004; Scarano et al., 2006). Poor sperm motility and density have also been observed in rats, diabetic mice and men (Amaral et al., 2006; Scarano et al., 2006).

Excessive levels of apoptosis are related to neurodegenerative processes and a decrease in the number of beta-pancreatic islet cells, which is thought to be an underlying factor in defective insulin secretion (Butler et al., 2003). Similarly, male mice with insulitis (an illness that shows the same symptoms as diabetes I) have greater amounts of spermatocytes, spermatogonia and spermatids undergoing apoptosis, along with higher levels of phosphorylated JNK (c-Jun NH_2-terminal kinase), activated BAX and caspase-3 (Sainio-Pollanen et al., 1997; Koh, 2007). Taken together, these findings suggest the occurrence of apoptosis in the testes of diabetic rats.

Diabetes, Reactive Oxygen Species and Spermatogenesis

High levels of ROS have been found in the pancreas, liver and kidney of diabetic rats, and as well as the testes (El-Missiry, 1999; Shrilatha, 2007; Shrilatha and Muralidhara, 2007). Hyperglycaemia induces superoxide production by disrupting mitochondrial electron chain transport, which inhibits glyceraldehyde-3-phosphate dehydrogenase activity, creating a metabolic imbalance of ROS levels in the cells (Wentzel et al., 2003; Blatnik et al., 2008). On the other hand, high ROS levels could also be achieved by decreasing the antioxidant capability of the cell. Wistar rats treated with streptozotocin showed a reduction in catalase (CAT), GR, GPx and SOD after 1, 2, 4 and 6 weeks post-streptozotocin injection, while the activity of glutathione-S-transferase (GST) was significantly increased (Shrilatha, 2007). In

rabbits treated with alloxane the activities of CAT, GPX, GSH and GR were increased after 10 weeks of treatment (Gumieniczek and Wilk, 2009). A reduction in GPx but not in GR was also reported in Goto-Kakizaki rats, which are currently being used as an animal model for type 2 diabetes. Cytosolic and mitochondrial fractions of diabetic adult Wistar rat testes were found to have higher levels of lipid peroxidation, as determined by the Thiobarbituric Acid Reactive Substances (TBARS) assay, and considerable amounts of ROS compared to healthy rat testes (Amaral et al., 2006; Shrilatha and Muralidhara, 2007). In addition, levels of advanced glycation end (AGE) product adducts and their receptors (RAGE) are higher in the reproductive tract of diabetic patients, and the presence of RAGE implies that they may play a role in sperm DNA damage (Mallidis et al., 2007).

In contrast, antioxidant therapy is recognized as counteracting the adverse effects of streptozotocin treatment. Antioxidants are chemicals that rapidly react with the unpaired electrons of ROS and RNS, preventing the harmful actions of these free radicals. The reproductive functioning of diabetic rats was found to be restored by the administration of C_{60} fullerene in drinking water (concentration ~30 nM) for 5 weeks (Bal et al., 2011). The authors found that this antioxidant improved sperm motility, epididymal sperm concentration, and the weights of the testis, epididymis, vesicula seminalis and prostate in diabetic rats. Furthermore, haematoxylin-eosin-stained sections of diabetic rat testes treated with C_{60} fullerene showed that the histological appearance was very similar to that of healthy rats. In other study, the activity of antioxidant enzymes in mice testes increased with the administration of daily injections of ascorbic acid compared to diabetic rats with no antioxidant treatment (Shrilatha, 2007).

GLUCOSE AND SPERMATOGENIC CELLS

Spermatogenic cells develop in a protected environment. On one hand, the lower temperature of the testis provides as yet poorly defined biochemical and physiological conditions for testicular functioning and spermatogenic cell development and, on the other, Sertoli cells provide a compartmentalized microenvironment (adluminal) for the development of meiotic and post-meiotic spermatogenic cells (Mruk and Cheng, 2004; Yan et al., 2008). The ionic, substrate and growth and death factor compositions of the adluminal compartment are still a mystery. Only a few hints have been obtained from the analysis of seminiferous tubule fluid obtained by micropuncturing (Tuck et al., 1970; Levine and Marsh, 1975; Mruk and Cheng, 2004), from the *in vitro* release of compounds by Sertoli cells in culture (Jegou, 1993) and from transport measurements (see below) and the localization of transporters on Sertoli and spermatogenic cells (see below). Regarding glucose, no reliable data are available on the concentration of this substrate in the adluminal compartment, although data from Turner et al. (1980) and Angulo et al. (1998) strongly suggest that glucose can cross the haemato-testicular barrier toward meiotic and post-meiotic spermatogenic cells. Glucose can be transported into spermatogenic cells (Rauch et al., 2006) and be metabolized by these cells. In fact, the metabolism of glucose by these cells was reported to induce a fall in intracellular ATP and an increase in intracellular AMP in round spermatids (Grootegoed et al., 1986; Nakamura et al., 1986; Reyes et al., 1990). Along with these adenine nucleotide changes, an increase in $[Ca^{2+}]i$ and a decrease in pH were reported (Herrera et al., 2000;

Reyes et al., 2002). These changes in $[Ca^{2+}]i$ were enough to sensitize plasma membrane Ca^{2+} channels to opening by extracellular stimuli (Reyes et al., 2010). These effects of glucose were even produced at low glucose concentrations (0.5-1 mM), strongly suggesting that these glucose-driven short-term physiological changes could occur under normal glucose concentrations in the plasma. Although neither normal nor diabetic concentrations of glucose have been reported in seminiferous tubules, the glucose-driven changes in spermatogenic cells described above are clearly similar to the pro-apoptotic conditions (especially if chronically maintained) often found in cells and specifically described in apoptotic spermatogenic cells (Lizama et al., 2007). Furthermore, our unpublished data indicate that, in the absence of lactate, glucose induces apoptosis in cultured rat spermatocytes through an increase in ROS and intracellular calcium (Bustamante-Marín X and Moreno R, unpublished data). Therefore, glucose by itself seems to be an inducer of apoptosis in germ cells.

Taken together, these results suggest that an imbalance in ROS production (oxidative stress) has a role in the impairment of testicular function observed in animal models of diabetes, and it probably has a role in humans who have this pathology. However, other factors such as endocrine disorders or nutritional status must be taken into account to fully understand the complexity of these testicular pathologies.

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