Causes, effects and molecular mechanisms of testicular heat stress

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Abstract  The process of spermatogenesis is temperature-dependent and occurs optimally at temperatures slightly lower than that of the body. Adequate thermoregulation is imperative to maintain testicular temperatures at levels lower than that of the body core. Raised testicular temperature has a detrimental effect on mammalian spermatogenesis and the resultant spermatozoa. Therefore, thermoregulatory failure leading to heat stress can compromise sperm quality and increase the risk of infertility. In this paper, several different types of external and internal factors that may contribute towards testicular heat stress are reviewed. The effects of heat stress on the process of spermatogenesis, the resultant epididymal spermatozoa and on germ cells, and the consequent changes in the testis are elaborated upon. We also discuss the molecular response of germ cells to heat exposure and the possible mechanisms involved in heat-induced germ cell damage, including apoptosis, DNA damage and autophagy. Further, the intrinsic and extrinsic pathways that are involved in the intricate mechanism of germ cell apoptosis are explained. Ultimately, these complex mechanisms of apoptosis lead to germ cell death.

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Introduction

The lack of thermoregulation of scrotal temperature causes testicular hyperthermia, which leads to genital heat stress. This is detrimental to spermatogenesis and results in spermatozoa of inferior quality. Both the epididymal sperm and testicular germ cells are sensitive to damage by heat stress (Zhu et al., 2004). Spermatozoa resulting from sperm cells exposed to hyperthermia in mice undergo apoptosis (Yin et al., 1997b) and contain damaged DNA (Perez-Crespo et al., 2008), leading to poor fertilizing capacity in vivo and in vitro (Yaeram et al., 2006).

Significant apoptotic loss of germ cells after testicular heat stress may occur either through intrinsic or extrinsic pathways. The molecular events that arise in germ cells exposed
to heat stress include the pro-apoptotic Bax and anti-apoptotic Bcl-2, cytochrome C, caspases and other heat-induced factors (Kim et al., 2013). The germ cell apoptosis response that follows heat stress takes place in a developmental stage-specific manner, with the spermatocytes (diplotene and pachytene) and spermatids being most prone to heat-induced changes (Lue et al., 1999; Setchell, 1998). The reason for this vulnerability, however, has not been elucidated.

The severity of damage to sperm cells subjected to heat stress varies with the intensity, frequency and duration of heat exposure (Collins and Lacy, 1969; Paul et al., 2008). When germ cell apoptosis occurs it is also influenced by the severity and duration of heat stress (Kim et al., 2013).

In this review, the following are discussed: the effects of hyperthermia on spermatogenesis, the measurement methods of scrotal temperatures, the various modifiable and non-modifiable factors that could cause increased testicular temperatures, the molecular mechanism of apoptosis, DNA damage and autophagy, changes in gene expression and the pathways of germ cell apoptosis in response to testicular heat stress.

**Effects of heat stress on spermatogenesis and testis**

**Testicular thermoregulation**

For optimal spermatogenesis to occur, testicular temperatures are maintained 2–4°C lower than core body temperature (Mieusset and Bujan, 1995). The temperature within the testes is reflected by the temperature of the surrounding scrotal sac. Thermoregulation of the testis is aided by several characteristics of the scrotal sac, such as thin skin with minimal subcutaneous fat, dense sweat glands and scant hair distribution. The musculature and vasculature in the genitals play a role in regulating testicular temperature as well. To maximize heat loss, the cremaster muscle that surrounds the testes and spermatogenic cords and the dartos muscle that lies beneath the scrotal skin relax, causing the testes to hang away from the abdomen and the scrotal skin to slacken, increasing the total surface area for easy heat dissipation. Further, vaso-dilation of scrotal vessels and activation of sweat glands promote heat loss when temperatures increase.

The testis is also thermoregulated via the counter-current mechanism. Testicular artery and veins facilitate heat exchange from the ‘warmer’ inflowing arterial blood to the cooler outgoing venous blood. This transfer of heat assures that ‘cooler’ arterial blood reaches the testis while the ‘warmed’ venous blood disperse heat through the thin, scrotal skin (Glad Sorensen et al., 1991). Testicular veins carrying the ‘warm’ blood anastomose and drain into the pampiniform plexus. In the case of varicocele, the pampiniform plexus becomes dilated, causing stasis and backflow of ‘warm’ blood back into the internal spermatic veins. The compromised counter-current heat exchange thereby contributes to the increased testicular temperatures found in varicocele patients (Setchell, 1998).

**Spermatogenesis**

The developmental process of the male gamete involves spermatogenesis in the lumen of the seminiferous tubules in the testis, followed by spermiogenesis in the epididymis. Human spermatogenesis requires almost 74 days for a complete cycle, whereas sperm cells complete epididymal maturation in about 12 days. The sequential cellular events of the spermatogenetic process initiate at the basal compartment and conclude at the apical compartment of the seminiferous tubules. Testosterone plays a crucial role in maintaining normal spermatogenesis at the seminiferous tubules.

**Events at the basal compartment of the seminiferous tubules**

The two main events that occur here are during spermatogoniogenesis (type $A_{pale}$ spermatogonia renew itself to generate the stem cell pool), and type $A_{pale}$ (spermatogonia develop into Type B spermatogonia), which progress to preleptotene, followed by lepotetne primary spermatocytes.

**Events at the apical compartment of the seminiferous tubules**

The three main events that occur here are spermatocytogenesis (primary spermatocytes progress from zygotene, pachytene, diplotene stages, to secondary spermatocytes then haploid spermatids); early round spermatids develop into elongated spermatids and undergo spermiogenesis to form spermatocyte with fully compacted chromatin; and spermiation (maturation and subsequent release of spermatocyte into seminiferous lumen).

**Spermiation and maturation in the epididymis**

Once in the lumen, spermatocytes leave the testis through rete testis into the epididymis, where sperm cells increase in concentration (caput), undergo maturation (corpus), and are stored (cauda). Sperm cells at the tail end of the epididymis have achieved full maturation, fertilizing ability and motility.

**Naturally occurring defects during spermatogenesis**

The spermatogenic potential for human reproduction is a mere 12%, as the remaining sperm cells that develop either degenerate, undergo apoptosis or develop abnormally (Sharpe, 1994). Defects may occur during any part of spermatogenetic process. In spermatogoniogenesis, failure of $A_{pale}$ spermatogonia to develop into Type B spermatogonia leads to spermatogenic arrest (Holstein et al., 1988). During a defective meiotic phase, apoptotic spermatocytes and spermatogenic arrest of primary spermatocytes may occur. The cytoplasm (cytoplasmic droplet) of the immature sperm cell is eliminated in spermiation, however, in some defective spermatozoa, the cytoplasm may still remain as excess residual cytoplasm (Breucker et al., 1985). During spermiogenesis, when haploid spermatids transform into fully differentiated spermatozoa, defects that may occur (which are likely attributable to genetic factors) include absence of the acrosome, absence of the midpiece of the flagellum and damaged nuclear condensation (Holstein et al., 2003). Defects in the morphology of the ejaculated spermatozoa (head, neck, midpiece, tail, or all) are commonly seen during analysis of the seminal fluid (WHO, 2010).
Spermatogenesis, however, may be interrupted partially or completely by factors such as oxidative stress, increased scrotal temperature, nutritional and hormonal imbalance, side-effects of therapeutic drugs and radiation, which will produce defective spermatozoa.

**Effect of heat stress on sperm cells and the testis**

Testicular thermoregulation is important to maintain testicular temperature within an optimal range for spermatogenesis. Increases in scrotal temperature, albeit within physiological range, negatively affect sperm quality (Hjollund et al., 2000). An increase of 1 °C entails a 14% drop in spermatogenesis, and consequently poorer sperm production (Wang et al., 1997). The effect of temperature on male fertility is evident with the mean scrotal temperature in infertile men being higher than fertile men and the quality of sperm deteriorating further with higher increases in scrotal temperature (Mieusset et al., 1987).

Spermatogenesis, especially the differentiation and maturation of spermatocytes and spermatids, is temperature-sensitive. Spermatogenesis should occur ideally at a minimum of 2 °C below core body temperature (Chowdhury and Steinberger, 1970; Thonneau et al., 1998). Elevated scrotal temperature, however, causes testicular germinal atrophy, spermatogenic arrest (Munkelwitz and Gilbert, 1998) and decreased levels of inhibin B (a biochemical marker of spermatogenesis) (Jensen et al., 1997), which leads to lower sperm counts (Hjollund et al., 2002a).

**Effects of heat stress on germ cells**

Germ cells are more vulnerable to heat stress as they have high mitotic activity (Shiraishi et al., 2012). Among the germ cells, the types that are most vulnerable to heat are the pachy- tene and diplotene spermatocytes and the early round spermatids in humans (Carlson et al., 2003) and rats (Chowdhury and Steinberger, 1970; Lue et al., 1999) alike. The basic mechanisms with which germ cells incur damage include germ cell apoptosis (Lue et al., 1999, 2002; Yin et al., 1997b) and autophagy (Eisenberg-Lerner et al., 2009; Zhang et al., 2012), damaged DNA due to altered synapsis and strand breaks (Shikone et al., 1994; Yin et al., 1997b), and generation of reactive oxygen species (Ahotupa and Huhtaniemi, 1992; Ikeda et al., 1999; Peltola et al., 1995). The molecular responses of germ cells of the hyperthermic testis will be further elaborated in the section Molecular response of male germ cells to heat stress.

**Effects of heat stress on epididymal sperm**

Epididymal sperm are affected by heat exposure differently from germ cells. Male mice that were whole body-exposed to temperatures of 36 °C for 12 h on two successive days were found to have lower sperm count, lower testicular weight, lower sperm fertility capacity in vivo and produced smaller litter sizes compared with controls. Epididymal spermatozoa from the heated mice had lower sperm-zona pellucida binding and oocyte penetration capacity. These effects were first seen at 1 week, and became more prominent at 2 weeks after heat exposure (Yaeram et al., 2006). In another study, sperm obtained from the cauda epididymis of mice exposed to whole-body heat (37 °C to 38 °C × 8 h × 3 consecutive days) had similar sperm count but lower motility. These epididymal sperm also exhibited membranous changes, making these sperm more prone to apoptosis (Wechalekar et al., 2010). In a previous study, when heat shock was applied to male mice at 42 °C for 30 min, the resultant spermatozoa retrieved from the epididymis demonstrated reduced count, motility and viability. Spermatozoa that resulted from heat-exposed spermatocytes and spermatids both had damaged DNA, with the spermatozoa from heat-exposed spermaticids showing poorer DNA integrity (Perez-Crespo et al., 2008).

**Other changes in the testis**

Shortly after heat exposure, a loss in testicular weight occurs. This reduction in testicular weight can be ascribed to germ cell loss, mainly by apoptosis. Although testicular weight may be partially regained several weeks after heat exposure in the rat, the testis remains lighter than before (Setchell et al., 2002). Microscopic examination of the rat testis showed mitochondrial degeneration, dilatation of the smooth endoplasmic reticulum, and wider spaces in both Sertoli and spermatid cells, after heat exposure (Kanter et al., 2013). The somatic cells of the rat testis such as the Sertoli cells (Cai et al., 2011) and Leydig cells (Kanter and Aktas, 2009) are also affected by heat stress, which renders them unable to provide a supportive role to germ cell development.

**Measurement of scrotal temperature**

The testis and epididymis represent the major thermal mass in the hemiscrotum, and intrascrotal skin surface temperatures reflect the temperature of the underlying testis (Zorgniotti, 1991; Zorgniotti and Macleod, 1973). When measuring testicular and intra-scrotal temperatures, accuracy and reproducibility is essential as temperature differences in a euthermic and hyperthermic testis may be as little as 0.6–1.4 °C (Zorgniotti and Macleod, 1973). Ideally, instruments used must be well-calibrated, have an accuracy of at least ±0.1 °C, allow for fast and easy temperature measurement in different body positions as well as repeated measurements on the same area (Zorgniotti, 1982). Depending on the method of measurement and presence or absence of pathology, testicular temperature may range between 31.0 °C to 36.0 °C. Testicular temperature may be taken as single or continuous measurements. Single measurements of intrascrotal temperature are made using a mercury thermometer (clinical thermometers are inadequate for this purpose), skin surface thermocouples (for measurement in a clothed state, provided the thermocouples stay in place), thermistor needles (invasive method that directly measures intrascrotal temperatures), infrared thermometry and thermography (a non-contact method measuring scrotal skin heat emission or thermal radiation and not deep scrotal temperature) (Zorgniotti, 1982), and liquid crystal thermometry (Zorgniotti et al., 1982). The most widely used, repeatable method for single measurement is the invagination method using a mercury thermometer (Brindley, 1982; Zorgniotti and Macleod, 1973). In this method, the patient is asked to disrobe from below the waist and lay supine for equilibration to ambient room temperature before measurement. Each testicle is measured
Factors that contribute towards testicular heat stress

Maintaining a temperature difference between the body and testes is crucial to ensure the production of normal spermatozoa. In daily life, however, a multitude of external and internal factors could narrow this temperature difference, thereby increasing the risk of abnormal spermatogenesis and the changes associated with increased testicular heat exposure. These thermogenic factors can be broadly grouped into lifestyle and behavioural factors, occupational and environmental factors (external factors) and clinical factors resulting from pathophysiological conditions (internal factors).

Lifestyle and behavioural factors

Lifestyle and behavioural causes of testicular heat stress encompass modifiable factors that are a result of habit or practices that could be altered or avoided with conscious effort.

Clothing and posture

Testicular temperature depends on scrotal position, which differs with postural changes. Scrotal temperature is at its lowest on an unclothed, upright body (Rock and Robinson, 1965; Zorgniotti and Macleod, 1973) as the position of the uncovered, hanging testis allows for easy heat dissipation. Scrotal temperatures are lower when walking compared with sitting because scrotal movement during ambulation provides better air circulation and heat dispersion. Accordingly, testicular temperature increases when movement is minimized, as in cases of prolonged sitting (testis cradled between the thighs) or lying down (scrotum resting on thighs) (Brindley, 1982; Rock and Robinson, 1965). After at least 20 min of being seated, male paraplegics in wheelchairs (approximated, unmoving thighs) had higher deep scrotal temperatures and less motile sperm compared with able-bodied men who sat freely (Brindley, 1982). Later studies differ in their results relating scrotal temperatures with impaired sperm quality in individuals with spinal cord injury (SCI). Wang et al. (1992) reported a higher initial scrotal temperature in men in wheelchairs with SCI compared with healthy men, attributing this to poor local thermoregulation owing to physical movement constraints. Conversely, Brackett et al. (1994) reported that scrotal temperature was not the leading cause of abnormal sperm quality (particularly sperm motility) in men with SCI, as men with SCI who walk (and do not use a wheelchair) also had poor semen quality.

A predominantly sedentary or seated position during long stretches of passive tasks such as working at the computer or commuting increases scrotal temperatures (Buñan et al., 2000; Hjollund et al., 2002b). The position of one’s legs and the type of chair used also influence scrotal temperatures. For example, sitting cross-legged on a typical cushioned office chair is likely to generate more scrotal heat compared with sitting on a saddle seat with wide-angled hips and knees, as the latter position promotes perigenital ventilation (Koskelo et al., 2005; Mieusset et al., 2007). Heat from the seated surface, as from a heated car seat or a heated floor, further adds to scrotal temperatures that are already elevated from being in a seated position (Jung et al., 2008a; Song and Seo, 2006).

Layers of clothing and bedding trap additional layers of air and impede air exchange, thus conserving heat and increasing scrotal temperatures. Being clothed elevates scrotal temperatures by 1.5–2°C when standing or supine, compared with an unclothed state (Mieusset et al., 2007; Zorgniotti et al., 1982). Thus, a choice of clothing that encourages good air flow could minimize the deviation of physiological scrotal temperatures. In this vein, the Scottish kilt (Kompanje, 2013) and the Asian sarong minus underwear would seem an ideal choice of leisure clothing, as the regular use of tight underwear was found to reduce sperm quality (Laven et al., 1988; Lynch et al., 1986; Tiemessen et al., 1996). Although the effect of tight underwear versus boxer shorts on sperm parameters is inconclusive, it would seem that tighter-fitting undergarments would leave less room for scrotal movement and air circulation hence contributing to higher genital temperatures (Munkelwitz and Gilbert, 1998).

Hot baths and sauna

The use of hot baths and sauna for relaxation and rejuvenation may make one feel better, but also has a negative effect on semen quality. Full-body immersion in a warm bath, hot tub, heated Jacuzzi or whirlpool at temperatures over 36.9°C for 30 min or more a week for 3 months or more leads to wet hyperthermia, which could have a reversible negative effect on sperm motility (Shefi et al., 2007). Users of typical saunas experience wet heat and warmed surfaces, whereas modern infrared-type saunas offer dry, radiant heat. Studies show that, after sauna exposure, scrotal temperatures reach up to body temperatures within 10 mins, and there is a significant but reversible negative effect on spermatogenesis (Jockenhovel et al., 1990). In saunas with temperatures ranging from 80–90°C, and at different frequency and duration of exposure, the use of saunas could disrupt spermatogenesis and cause abnormal sperm count and motility (Brown-Woodman et al., 1984; Garolla et al., 2013; Saikhun et al., 1998). Further, regular sauna exposure over an entire spermatogenic cycle also modified mitochondrial function, chromatin prolamination and condensation in the sperm (Garolla et al., 2013).
Laptop use

In the current online information era, the use of laptops is prevalent, and especially so in those within the reproductive age. As a computer on the lap, it is close to the genital area, and sitting with one’s legs close together for long hours increases scrotal temperature, which may negatively affect sperm parameters (Sheynkin et al., 2005).

Cycling

Among the different types of exercise, cycling is one that is reputed to impair male fertility. Aspects of cycling that may influence scrotal temperatures include posture, duration and intensity of cycling (Jung et al., 2008b), and the attire. Particularly in professional cyclists, extended periods of cycling in form-fitting spandex outfits and being seated for long hours on a saddle seat is likely to cause elevated scrotal temperatures (Lucia et al., 1996).

Obesity

Obesity is on the rise globally, and men with an above-normal body mass index (BMI) (>25) have an average of 25% lower sperm count and motility compared with men with a normal BMI (Kort et al., 2006). Not surprisingly, obesity rates tend to be higher in infertile men compared with men with euspermia (Hammoud et al., 2008). Obese men have compromised testicular thermoregulation owing to several factors: decreased physical activity and prolonged sedentary periods, increased fat deposition in the abdominal, suprapubic, spermatic cord (scrotal lipomatosis) and upper thigh areas, which leads to suppressed spermatogenesis (Ivell, 2007; Shafik and Olfat, 1981a, 1981b). About 65% of infertile patients whose excess scrotal and suprapubic fat were removed showed improvement in their sperm count, motility and morphology, with one in five of these patients successfully achieving a good pregnancy outcome (Shafik and Olfat, 1981b).

Occupational and environmental factors

Job exposure to heat-generating conditions are significant contributors to heat stress, especially as work takes up a substantial portion of the day and exposure to the source of radiant heat is likely to occur almost daily over long periods of time. Another factor that may contribute to heat stress in the male is ambient heat caused by hotter environmental temperatures where the men live.

Radiant heat

Certain labour-intensive jobs entail exposure to long periods of intense, radiant heat. Welders, for example, are exposed to strong levels of heat, toxic metals and fumes during welding. Studies involving these workers demonstrate reversible decline in semen quality (Bonde, 1992; Kumar et al., 2003). Those working directly with sources of severe heat, such as bakers and ceramic oven operators, have a longer time to pregnancy, which suggests that occupational heat exposure has an effect on fertility (Figa-Talamanca et al., 1992; Thonneau et al., 1997). Men who work in close range to sources of intense heat, such as the rear end of a submarine (location of motor) seem to face infertility-related problems (Velez de la Calle et al., 2001).

Professional or occupational drivers and individuals who have long daily commutes are more prone to having increased scrotal temperatures (Bujan et al., 2006), poorer sperm quality (Chia et al., 1994; Figa-Talamanca et al., 1996; Henderson et al., 1986; Sas and Szollosi, 1979) and longer time to pregnancy (Thonneau et al., 1996). The negative effect of long hours of driving and seated commutes increases in severity with the number of years spent engaging in such activities (Figa-Talamanca et al., 1996; Sas and Szollosi, 1979).

Ambient heat

A study on fertile, European men living in different cities reported a general seasonal variation in sperm concentration and total sperm count, with summer values being approximately 70% of their winter values. Their sperm motility and morphology, however, did not seem to vary with these seasons (Jorgensen et al., 2001). Other studies have shown a similar effect of changing seasonal temperature on sperm counts of healthy men (Gyllenborg et al., 1999; Tjoa et al., 1982), but this possible connection between seasonality and sperm concentration was not seen in healthy Australian men (Mallidis et al., 1991).

Clinical factors

Testicular hyperthermia resulting from pathological failure of thermoregulation imposes adverse effects on the spermatogenic process. Abnormalities such as cryptorchidism and varicocele result in exposure of the testis to raised temperatures and compromised sperm quality, which may lead to a loss in male fertility.

Cryptorchidism

Failure of the testis to fully descend into the scrotal sac before birth or within the first few months of life could lead to subfertility and increased risk of testicular germ cell cancer (Toppari et al., 2014). An undescended testis is exposed to body temperatures and, although painless, it could lead to heat-induced changes in the spermatogenic process, which affects the germ cells, spermatooza, testis and testicular hormones (Bertolla et al., 2006; Lee and Coughlin, 2002; Li et al., 2006; Liu and Li, 2010). The severity with which cryptorchidism affects fertility depends on whether one or both testes had failed to descend fully, its position along the inguinal canal, the cause of the incomplete descent and the length of time before surgical intervention to reposition the affected testicle into the scrotal sac (Agoulnik et al., 2012).

Varicocele

Varicocele affects 15% of the male population, 40% of men with primary infertility and eight out of 10 men with secondary infertility. It is the most commonly occurring and treatable cause of male infertility. Varicocele occurs when abnormal dilatation of the veins of the pampiniform plexus that surrounds the vas deferens in the spermatic cord occurs, which leads from the scrotum to the inguinal canal. The resulting backflow and stasis of blood hampers the counter current heat exchange between testicular arterial and venous blood, which consequently exposes the testis to temperatures closer to the core body temperature (Setchell, 1998).
The temperature of a testis affected with varicocele is about 2.5 °C higher than a normal testis (Goldstein and Eid, 1989) and in an infertile individual, scrotal hyperthermia is most likely varicocele-related (Mieusset et al., 1987). Higher scrotal temperatures in varicocele patients with infertility lead to sperm DNA fragmentation and apoptosis, as well as hormonal imbalance (Ku et al., 2005; Shiraishi et al., 2010).

**Febrile episodes**

During the onset of a fever, testicular thermoregulation is disrupted and scrotal temperature is raised along with the core body temperature. Fever, which lasts for a day or longer, affects the ongoing spermatogenic process. The magnitude of the temperature difference, duration (acute or prolonged) and timing (which stage of the spermatogenic cycle) of the febrile episode causes varying effects on the resultant spermatozoa (Carlsen et al., 2003; Evenson et al., 2000). Higher temperature differences, longer periods of temperature dysregulation, or both, result in a more severe effect on spermatogenesis. Further, particular stages of spermatogenesis seem more vulnerable to raised temperatures than others (Carlsen et al., 2003). For example, fever for more than 1 day that occurred when ejaculated sperm was undergoing spermiogenensis (9–32 days before ejaculation) caused a decrease in sperm concentration (by 35%) and normal morphology (by 7.4%); as well as an increase in percentage immotile sperm (by 20.4%). Fever during mitotic proliferation (57–80 days before ejaculation), meiosis (33–56 days before ejaculation) and sperm maturation (up to 8 days before ejaculation) did not significantly affect these parameters, except for reduced sperm concentration when fever occurred during meiosis (Carlsen et al., 2003).

In summary, at any one time, many factors contribute to testicular temperatures: posture, clothing, type of activity, occupational and environmental conditions as well as medically related afflictions. For example, using a quilt over typical nightclothes while lying down in bed after a hot bath, or a patient with a varicocele sitting with legs close together in well-fitting clothes on a couch with a laptop on the lap, will give a cumulative effect resulting in increased genital heat of the individual. Depending on the circumstance, genital heat exposure may be transient, prolonged or sustained with varying degrees of intensity. The damaging effects of heat exposure on sperm parameters and male fertility tend to accumulate with repeated exposure over a period of time.

**Molecular response of male germ cells to heat stress**

Several studies (mainly using the cryptorchid model) have investigated the molecular aspects of male germ cell apoptosis after heat stress, and these findings are elaborated upon in this section.

**Germ cell apoptosis**

Many experiments inducing cryptorchidism in animals have shown an increase in apoptosis of germ cells, probably caused by DNA damage, resulting in a decline in testis weight and infertility problems (Banks et al., 2005; Setchell, 1998; Yin et al., 1997b). Apoptosis, also known as type I programmed cell death, involves identifiable cellular changes, such as DNA fragmentation, cell volume shrinkage, and plasma membrane blebbing (Liu, 2010). During germ cell development, physiological germ cell apoptosis occurs to maintain the quality of the germ cell. Also, in the male germ cell, particularly, DNA damage is a precursor to apoptosis (Shaha et al., 2010). Henriksen et al. (1995) suggested that rat germ cells with heat-damaged DNA were being eliminated via apoptosis. Using histochemical staining, Yin et al. (1997b) found evidence of apoptosis, especially among primary spermatocytes and round spermatids, and the effect was more noticeable in more mature rats. Lue et al. (1999, 2002) used the newer technologies of TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling assay and electron microscopy to further confirm that rat and monkey germ cells were dying via apoptotic mechanisms. In addition to direct spermatozoa, DNA damage, heat may also denature cytoplasmic bridges necessary for cell survival and affect fluid composition in the cauda epididymis, which hinders proper spermatozoa maturation, thus contributing to the increase in apoptosis, both in rats and humans (Legare et al., 2004; Rockett et al., 2001). This is known as the heat shock response, which is facilitated by a group of proteins called the heat shock proteins (HSP) (Izu et al., 2004; Widlak et al., 2007).

There are two types of HSP: constitutive and inductive. Under normal circumstances, constitutively produced HSP are molecular chaperones that ensure polypeptides are assembled and transported correctly. They also contribute to other cellular processes, such as stabilizing proteins in their inactive forms and inhibiting degradation (Neuer et al., 2000; Son et al., 1999). On the other hand, as part of an innate protective mechanism conserved through evolution, cells respond to heat stress by halting the synthesis of most proteins and diverting all resources available to produce inducible HSP (Neuer et al., 2000; Pei et al., 2012; Widlak et al., 2007). These proteins tend to oligomerize in order to carry out their functions effectively (Sreedhar and Csermely, 2004). They protect cells from heat stress by binding to proteins and preventing their denaturation and incorrect folding. The extent of induction is dependent on the intensity and duration of heat exposure – the higher the temperature and the longer the exposure, the greater the amount of HSP produced to protect the cell. Because of its important function in ensuring correct assembly and transport of proteins, as well as protecting the cell against external stress, HSP are essential for spermatocytes to develop into healthy mature spermatozoa (Legare et al., 2004).

Heat shock factor 1 (HSF1), a protein that is produced intracellularly in sperm cells when the HSF1 gene is activated by heat stress, has two paradoxical roles (Izu et al., 2004). First, it is responsible for the rapid increase in HSP produced, thus promoting cell survival (Widlak et al., 2007). Conversely, it is also involved in directly eliminating aberrant germ cells to ensure that mature cells are of good quality (Izu et al., 2004; Widlak et al., 2007). The most common HSP activated by HSF1 is HSP70, which is found in elevated amounts in cryptorchid mice and rabbit testes (Pei et al., 2012; Rockett et al., 2001). HSP70 not only acts as a regulator of p53, a tumour-suppressor protein involved in extrinsic apoptosis, but is also involved in intrinsic apoptosis by causing an increase in cytochrome C necessary for caspase (cysteine–aspatic
protease) activation (Sreedhar and Csermely, 2004; Widlak et al., 2007). Caspases are crucial mediators of apoptosis and can be divided into two groups: initiators and apicals and executors and effectors. Although the former are responsible for starting the apoptotic process, the latter are in charge of carrying apoptosis out via cleavage of various proteins (Vera et al., 2004). Moreover, HSP70 is purported to play a vital role in the response to oxidative stress as well (Sreedhar and Csermely, 2004). These apoptotic and oxidative stress pathways will be further elaborated upon in the section Molecular mechanism of heat stress – proposed pathways.

Sperm DNA damage

Despite the high rate of apoptosis, some cells are usually able to survive cryptorchidism, but eventually develop into mature spermatozoa containing damaged DNA in murines (Banks et al., 2005). This could partly be due to the protective effect of inducible HSP, which bind to proteins, thus preventing their denaturation and incorrect folding (Legare et al., 2004; Widlak et al., 2007). Shikone et al. (1994) and Yin et al. (1997b) both detected DNA fragmentation in cryptorchid mice testes, and this is likely to be the cause of infertility (Banks et al., 2005; Shikone et al., 1994; Yin et al., 1997b). In rodents, chromatin structure was also found to be altered and chromatin material reduced (Blackshaw and Hamilton, 1970; Sailer et al., 1997). This loss of integrity is generally attributed to oxidative stress, but could also be due to defective repair mechanisms or destruction of the testes in murines such that spermatocytes are unable to develop properly (see section ‘Molecular mechanism of heat stress: proposed pathways’) (Banks et al., 2005; Setchell, 1998).

Increase in chromosomal abnormalities in the form of X-Y bivalent dissociations during metaphase I were also noted in experiments conducted by Garriott and Chrisman (1980) and van Zelst et al. (1995), in which male rats and mice were exposed to hyperthermic conditions. This is because excessive heat prevents the synaptonemal complex from functioning normally, resulting in the formation of fewer and more distal chiasma. Hence, bivalents are less strongly held together and the resulting presence of unpaired Y chromosomes will cause spermatocytes to undergo apoptosis (Garriott and Chrisman, 1980).

In addition to damaging DNA, hyperthermia also causes a decrease in DNA synthesis and the degradation of many mRNAs and proteins necessary for cell survival (Izu et al., 2004; Nishimune and Komatsu, 1972; Tramontano et al., 2000). Acid phosphatase and amino-peptidase reactions, considered essential for lysosomal function and normal protein synthesis respectively, were also altered in rats and the presence of multinucleated giant cells additionally caused a cumulative decline in the number of viable mouse spermatozoa produced (Blackshaw and Hamilton, 1970; Waldbieser and Chrisman, 1986).

Autophagy

Finally, autophagy, commonly described as type II programmed cell death, could also be responsible for causing germ cell death. Autophagy is a process in which cells are phagocytosed by vesicles, degraded by lysosomes, and the resulting cellular components recycled for energy generation (Eisenberg-Lerner et al., 2009; Zhang et al., 2012). When this pathway is activated by heat stress, the cytosolic form of light chain 3 (LC3B-I) is modified into a membrane-bound form (LC3B-II), and the ubiquitin-like conjugation system is activated as well. The formation of LC3B-II assists in proper development of autophagosomes, and the autophagosome formation by the conjugation system, both considered markers of the autophagy process (Zhang et al., 2012). Blackshaw and Hamilton (1970) also argued that the rapid DNA degradation and change in acid phosphatase and amino-peptidase reactions point to the involvement of lysosomes in the cellular response to heat stress in rats. Moreover, autophagy can work in concert with apoptosis as well – either together or as a back-up mechanism when apoptosis fails (Eisenberg-Lerner et al., 2009; Zhang et al., 2012).

Molecular mechanism of heat stress – proposed pathways

The aforementioned responses occur through various diverse pathways, and there may be some degree of crosstalk among them too (Paul et al., 2009). The pathways that will be explained in detail in this section are that of gene expression changes, stress response, impaired DNA repair as well as apoptosis (both intrinsic and extrinsic).

Gene expression changes

Heat stress alters the levels of expression of various genes in a complex manner. It is still debatable, however, if the modulation of gene expression levels are directly caused by heat stress or indirectly caused by other cellular changes (Setchell, 1998). Together with post-translational modification and protein localization, altered levels of gene expression will lead to changes in the protein composition of the spermatocyte (Kim et al., 2013). Overall, the changes in gene expression, as was seen in mice, indicate that the spermatocytes undergoing cellular shutdown in response to hyperthermia (up-regulated genes) have defensive or regulatory roles that are necessary for defense and repair, whereas all other genes are down-regulated (Rockett et al., 2001).

In addition to the up-regulation of HSF1 gene coding for HSF1 protein, which in turn increases HSP production, other genes that are up-regulated include those responsible for apoptosis, cell adhesion and signal transduction. For instance, in adult male mice, it was found that the expression of laminin and its receptors are up-regulated because laminin is required to maintain Sertoli cell barrier functions vital for proper spermatogenesis (Rockett et al., 2001). In mice, the phosho protein p53, which is involved in cell cycle arrest and promotes apoptosis when DNA damage is too extensive to be repaired, is also up-regulated to detect damaged cells and eliminate them (Absalan et al., 2012). Rockett et al. (2001) also highlighted that pro-apoptotic genes may be down-regulated in mice after a few hours of heat stress in order to prevent complete destruction of all cells.
One of the genes that were found to be down-regulated, Bag-1, prevents normal HSP70 functioning such that the proteins are not folded correctly. Thus, a decrease in Bag-1 would result in increased activity of HSP70 (Rackett et al., 2001). Banks et al. (2005) showed that levels of cold inducible RNA binding protein (Cirp) are decreased, hence cellular processes like mitosis and meiosis are uncontrolled and germ cells undergo apoptosis. Other genes such as DNA polymerase β and DNA ligase III, are down-regulated in male rats as well, preventing DNA repair from occurring and further contributing to apoptosis (Tramontano et al., 2000).

### Stress response pathway

Free radicals are molecules with at least one unpaired electron, making them extremely unstable and highly reactive (Sharma and Agarwal, 1996). They collide into neighbouring molecules and cause propagative chain reactions that produce even more free radicals (Tremellen, 2008). Reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hypochlorite radicals, are produced during oxygen metabolism and are found in the testes because they help in spermatozoal functions such as capacitation, acrosome reaction, hyperactivation, and sperm-oocyte fusion (Agarwal et al., 2012; Shiraishi et al., 2012). To maintain ROS at an acceptable level, natural antioxidants, such as vitamins C and E and carotenoids are present in the testes. When this balance of free radicals and antioxidants is upset, oxidative stress occurs and this results in apoptosis (Agarwal et al., 2012; Paul et al., 2009).

Reactive oxygen species are known to be produced in cryptorchid testes and testes with varicocele, and there are two probable ways in which they could be involved in the heat stress response. Firstly, oxidation of cellular components such as DNA and lipids could lead to apoptosis directly and, secondly, the generation of ROS could indirectly trigger the activation of apoptosis (Ishii et al., 2005; Shiraishi et al., 2010).

To support the first hypothesis of directly causing apoptosis, Ahotupa and Huhtaniemi (1992) and Ikeda et al. (1999) found that when rats’ testes were exposed to heat stress, an increase in hydrogen peroxide and, hence, lipid peroxidation was accompanied by a decrease in the activity of enzymatic antioxidants such as superoxide dismutase and catalase. As such, compromised antioxidant capabilities led to higher ROS concentrations and more oxidative stress. In fact, Ikeda et al. (1999) further demonstrated that treating the rats with catalase reduced the amount of peroxidation and apoptotic cells, therefore lending even more support to the hypothesis. The results of another experiment conducted by Peltola et al. (1995) in rats, however, suggested that the increased oxidative stress was more likely a result of the rapid increase in ROS rather than the decreased antioxidant capacity. This is because the observed decline in manganese superoxide dismutase was not consistent with the decrease in amount of normal DNA. This indicates that the decrease in antioxidants was not the main factor causing increased DNA damage and that the increase in ROS most likely had a more significant role. Regardless of the cause of increased oxidative stress, the peroxidation of cellular components is still likely to be involved in apoptosis.

As for the second postulation, Ishii et al. (2005) argued that the short duration of heat exposure (approximately 15 min), which caused damage to spermatocytes in SOD1-knockout mice, was an insufficient amount of time for any ROS generated to cause peroxidation and eventually apoptosis. Hence, it was more likely that the ROS produced acted as a signal to trigger apoptotic mechanisms.

#### Impaired DNA repair pathway

The effects of hyperthermia could also occur as a result of impaired DNA repair mechanisms. As gene expression levels of DNA polymerase beta and DNA ligase III levels reduce in response to heat exposure, the germ cell’s ability to repair its DNA decreases (Tramontano et al., 2000). Additionally, poly(ADP) ribose polymerase, another enzyme involved in DNA repair, is substantially decreased in cryptorchid rat testes (Tramontano et al., 2000). Banks et al. (2005), however, proposed an alternative theory, which states that DNA damage caused by excessive heat could be too great for the natural testicular repair mechanisms to cope with and thus, some but not all, of the DNA damage is repaired. As such, although still damaged, the DNA may persist into mature, motile spermatozoa. Thus, in an assisted reproduction technique setting, particularly in intracytoplasmic sperm injection cycles, spermatozoa selection should take into account the DNA integrity and its role in spermatozoa viability (Banks et al., 2005).

#### Apoptotic pathways

Heat stress results in the above mechanisms – gene expression changes, stress response and impaired DNA repair – which eventually lead to apoptosis of abnormal germ cells. Apoptosis can occur intrinsically or extrinsically (Figure 1).

### Intrinsic apoptotic pathway

The intrinsic apoptotic pathway, also known as the mitochondria-dependent apoptotic pathway, occurs in all cells and is triggered by the translocation of certain members of the Bcl-2 protein family, such as the pro-apoptotic Bax (Bcl-2-associated X protein) and anti-apoptotic Bcl-2 (B-cell lymphoma 2) (Liu, 2010). In healthy cells, Bax is largely found in the cytosol. In response to heat stress, however, Bax accumulates in the mitochondria and endoplasmic reticulum, whereas Bcl-2 localizes on the mitochondrial membrane (Hikim et al., 2003; Liu, 2010; Vera et al., 2004). The relocation of Bax is also coupled with the concentration of ultra-condensed mitochondria in paranuclear areas of spermatocytes destined for apoptosis (Liu, 2010; Vera et al., 2004). Although Bcl-2 is phosphorylated and thus inactivated, Bax is inserted into the outer mitochondrial membrane, which leads to conformational changes that allow the release of cytochrome C, into the cytosol (Hikim et al., 2003; Kim et al., 2013). Cytochrome C is a small protein that plays a major role in the electron transport chain, and is commonly found on the inner mitochondrial membrane taking part in redox reactions. In the cytosol, cytochrome C interacts with apoptotic protease activating factor 1 (Apaf-1) to form a complex (Hikim...
The activated form of Apaf-1 subsequently binds to initiator (apical) caspase 9 and proteolytically activates the caspase cascade via executioner caspases like caspases 3, 6 and 7 (Liu, 2010; Vera et al., 2004). These caspases are involved in the cleavage of various structural and repair proteins, such as poly(ADP) ribose polymerase, lamin and actin, resulting in morphological changes and eventually, apoptosis (Hikim et al., 2003; Vera et al., 2005). As previously mentioned, ROS possibly triggers this apoptotic pathway directly by causing the release of cytochrome C into the cytosol via altered functions of signalling molecules (Ishii et al., 2005).
Extrinsic apoptotic pathway

Two pathways have been proposed to explain how germ cells undergo apoptosis extrinsically: the Fas/Fas ligand (FasL) system or p53 system (Ishii et al., 2005; Izu et al., 2004).

Fas is a type I transmembrane receptor protein whereas FasL is a type II transmembrane protein (Miura et al., 2002). This death receptor pathway requires the ligation of the death receptor (Fas) to its ligand (FasL) to be initiated, which causes Fas to trimerize and recruit Fas-associated death domain (FADD) through shared death domains (DD) (Hikim et al., 2003; Vera et al., 2004). Activated DD subsequently triggers the caspase cascade when Fas/FADD complex binds to initiator/ apical caspases 8 or 10 with its N-terminal death effector domain (DED), and this results in the activation of executioner/effector caspases 3, 6 and 7 (Hikim et al., 2003; Miura et al., 2002; Vera et al., 2004). Hikim et al. (2003) and Vera et al. (2004) conducted studies on FasL-defective gld (generalized lymphoproliferation disease), or Fas-defective lprcg (lymphoproliferation complementing gld) mice, or both, and proved that heat stress-induced apoptosis still occurred. These results suggest that the Fas/FasL pathway is not necessary for apoptosis, and that another pathway involving p53 was also a key player in cell death.

The tumour suppressor p53 is a transcription factor that increases the expression of pro-apoptotic genes when exposed to heat stress and therefore regulates the cell cycle (Miura et al., 2002). In hyperthermic conditions, p53 is relocalized from the nuclear envelope into the nucleus, where it binds to DNA and causes cell cycle arrest or apoptosis (Yin et al., 1997a). Yin et al. (1997a) showed that apoptosis levels were lower and delayed, and the amount of DNA damage was higher, in mice lacking the p53 gene post-heat exposure. This implies that the p53 pathway is not the sole mechanism by which extrinsic apoptosis is carried out (Yin et al., 1998b).

It is, therefore, conceivable that both the Fas/FasL and p53 pathways work together to cause apoptosis. Miura et al. (2002) found that the amount of Fas dramatically increased within 1 day of heat exposure whereas p53 was only up-regulated after 3 days, thus suggesting that early apoptosis is Fas/FasL-mediated whereas late apoptosis is carried out via the p53 pathway. Conversely, Yin et al. (1998b) produced conflicting results, which indicated that p53 was in fact responsible for early apoptosis whereas Fas/FasL was involved in the later stages (Yin et al., 1998a). Further experiments are required to determine which system is responsible for which phase of apoptosis, but it is safe to conclude that both play an important role in the response to heat stress.

Crosstalk between various pathways

Furthermore, an extensive degree of crosstalk occurs among the various pathways (Liu, 2010; Vera et al., 2004). For instance, p53 up-regulates the activity of pro-apoptotic genes like Bax by promoting its insertion into the mitochondrial membrane, and down-regulates anti-apoptotic genes like Bcl-2, both of which are mediators of the intrinsic apoptotic pathway (Miura et al., 2002). Intrinsic and extrinsic pathways also converge on the downstream effector executioner caspases 3, 6 and 7 (Liu, 2010). As such, the mechanism of apoptosis is complex and can be carried out via different pathways, ultimately resulting in germ cell death.

Conclusion

Spermatogenesis involves a complex series of stages that involve the development of spermatogonia into specialized spermatozoa. The developmental processes of the male gamete may be influenced by various factors, including heat stress, causing the production of sperm with lower quality and thus affecting fertility. The factors that contribute to increased scrotal temperatures range from lifestyle, occupational, environmental to pathophysiological. Many of these factors can hardly be avoided altogether. Testicular thermo-regulation is therefore of great importance to ensure the production of viable spermatozoa and to maintain fertility. However, failure to regulate scrotal temperatures or exposure to high temperatures result in testicular heat stress. Sperm cells are vulnerable to heat stress and respond by undergoing apoptosis (germ cells) and DNA damage (both germ cells and epididymal sperm). Heat stress also modifies gene expression in the testis that could impair the regular spermatogenic processes. The consequences of testicular heat stress to the male germ cell are presented in Figure 2.

Consequences of heat stress on germ cells, however, are not thoroughly understood. This warrants further genetic studies to shed more light on pathways that regulate heat stress responses of male germ cells and discover new genes that may be involved. Understanding the molecular mechanisms of testicular heat stress would aid in developing targeted male infertility therapies and contraception. Meanwhile, both fertile and infertile men who are looking to start or continue their family may do well to refrain from as many different types of factors that induce heat stress as possible, so that harmful effects of hyperthermia on sperm quality can be minimized.

Figure 2. Autophagy and apoptosis: response of the male germ cell to testicular heat stress. Proposed pathways and the responses that lead to apoptosis. When the testicles are under heat stress, germ cells undergo apoptosis via the intrinsic or extrinsic mechanism. Along with, or as a back up to, apoptosis, autophagy is also responsible for germ cell death. Apoptosis can occur through several means, such as stress response, DNA damage and changes in gene expression, all of which result in impaired DNA repair that lead to germ cell death.
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