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Spermatogenesis, heat stress and male infertility

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HIGHLIGHTS

- Apoptosis and germ cell death are a normal part of spermatogenesis.
- Apoptosis of spermatocytes and spermatids increases during heat stress.
- Heat stress upregulates the heat stress factors (HSFs) HSF1 and HSF2 leading to prosurvival effects in testicular somatic cells and apoptosis in meiotic germ cells.
- Androgen production decreases and androgen receptor is downregulated in heat stress, impacting the blood-testis barrier (BTB) and support of meiosis.
- Unifying theories of heat stress now explain discrepancies in mild to severe heat stress in animal models.
- Both contraceptive research and improvement of sperm production have exploited research in heat stress.

17.1 INTRODUCTION

The production of sperm occurs within the testes of mammals. In most mammals, the testis is held in the scrotal sacks outside of the body cavity and thus is maintained 2°C–8°C below body temperature. In some mammals such as elephants, porpoises and whales, the testis is within the body cavity, and spermatogenesis may occur at higher temperatures near the core body temperature. Little is known about how spermatogenesis is regulated in such abdominal testes. This review focuses on mammals with external testes.

Once puberty is reached, spermatogenesis occurs continuously to produce sperm until a gradual decrease occurs in old age. Within the testes are two general spaces: the seminiferous tubules, which contain germ cells, and the Sertoli cells, which are somatic cells. The seminiferous tubules are surrounded by a basement membrane and contractile myoid cells. Outside the seminiferous tubule is the interstitial space containing Leydig cells, blood vessels, lymphatics and nerve cells. Myoid and Leydig cells are again somatic cells.

Germ cells go through mitosis as spermatogonia; meiosis as primary spermatocytes, secondary spermatocytes and round spermatids; and then spermiogenesis, which consists of a morphological change to form the spermatozoa (reviewed in [1]). The general sequence is that type A_1 spermatogonia divide mitotically three times to form 2 A_2, 4 A_3 and finally 8 A_4 spermatogonia, dividing again mitotically to form 16 intermediate spermatogonia and then 32 B spermatogonia, and finally 64 primary spermatocytes. The primary spermatocytes must then cross a junctional barrier known as the blood-testis barrier (BTB) that is composed of a tight junction, desmosomes, gap junction and ectoplasmic specializations (2). The basal compartment is between the BTB and the basement membrane, the adluminal compartment is between the BTB and lumen and then the lumen is in the center of the seminiferous tubule. Following mitosis in the basal compartment, meiosis occurs in the adluminal compartment in which the primary spermatocytes divide to form 128 secondary spermatocytes and then divide again to form 256 round spermatids. A modification to the round

spermatids occurs to transform them into spermatozoa with no further divisions in the process of spermiogenesis. The spermatozoa are then released into the lumen in spermiation. As a developing type A spermatogonia goes through these divisions producing the various daughter germ cells, it is associated with a single Sertoli cell that controls and coordinates the events of spermatogenesis. There is also a coordination along the length of seminiferous tubules consisting of waves and cycles that ensure a steady production of sperm. As a normal part of spermatogenesis, there is an overproduction of germ cells that if allowed to continue would disrupt the BTB and function of the seminiferous tubule. The processes of apoptosis and autophagy are thus normal events that limit the eventual number of spermatozoa produced (1,3–5). In porcine spermatogenesis, only 10%–30% of the potential spermatozoa are produced (1,6,7), while approximately 25% reach this point in rodent and human spermatogenesis (3).

Stressors are now known to exploit the decretory processes of apoptosis and autophagy during spermatogenesis (4). Many stressors disrupt thermoregulation of the testis, such as increased environmental temperatures, cryptorchidism, febrile disease, fever as a result of vaccination or obesity (4,8,9). In humans, this may also be as a result of lifestyle choices, but in livestock, it is human intervention from designs of housing, environmental controls or other management factors. The end result of these heat stressors is a decrease in the number and quality of sperm produced.

There have been many studies examining the molecular mechanisms of spermatogenesis (1–3,10,11). The goal has been to either understand the mechanisms that might be exploited for contraception or how to increase the number and quality of sperm produced. The former has focused on research related to rodent, nonhuman primate and even humans. The latter is work principally with livestock species such as porcine, bovine, ovine and even equine. The work with heat stress is converging on a unified theory of how apoptosis and other cell death pathways play a role in normal, pathological and environmental regulation of spermatogenesis.

17.2 HEAT STRESS

Studies in a variety of species come to the same conclusion about heat stress. First there is a decrease in the amount of sperm produced (1,4,10,12,13). This is associated with increased apoptosis of primary spermatocytes through round spermatids (14,15). Interestingly, even sperm that survive this apoptosis may have damaged DNA and are of lower fertility (16,17). Animal models have used increased environmental temperatures, mild local scrotal heating, experimental induced cryptorchidism or even short-term higher temperatures applied to the scrotum (1,18). Human exposure to testicular heat stress is usually via lifestyle choices, job-related conditions or pathological conditions (4). The intensity and/or duration of the heat exposure impact the severity of the response to spermatogenesis observed. For example, scrotal insulation can produce a rise in testicular temperatures close to body temperature and is similar to experimental cryptorchidism. Scrotal insulation for 48 hours can produce an immediate effect on testicular histology, abnormal sperm produce 3–5 weeks later, but following the normal length of spermatogenesis there is complete recovery to pretreatment levels of germ cell differentiation and production of sperm (1,16,17). A common approach in rodents that has also been applied to some livestock species is a short-term increase for 30–120 minutes in scrotal temperatures to 41°C–43°C, well above body temperature (12,19,20). This is a more severe procedure with spermatogenesis often completely disrupted, although in some cases, recovery can occur with very short heat exposure (12). Increasing environmental temperatures into the range of 35°C–37°C for several hours a day followed by returning animals to normal conditions of 21°C–23°C can also produce similar changes but requires exposure for most of the length of spermatogenesis and a range from 3 to 6 weeks to achieve consistent results (18,21). Changing environmental temperatures were intended to mimic changes seen during warmer months of the year, but just measuring semen or testicular tissue response to animals under summer conditions produces variable results, as conditions do not remain constant and may or may not increase in a particular year (1).

Most of our detailed knowledge of heat stress mechanisms of damage in the testis comes from rodent models. While both testicular and epididymal germ cells are sensitive to heat stress, we focus on the testicular cells. Germ cells and, in particular, primary spermatocytes undergo apoptosis, and if they survive will often contain damaged DNA and poor fertility when ejaculated as spermatozoa (22–24). This is also true in the bull (16,17). Both intrinsic and extrinsic pathways of apoptosis occur in response to heat stress in primary spermatocytes and round spermatids (4), as well as disruption to the BTB, which then also impact survival of primary and secondary spermatocytes, and spermatids (12). The intrinsic mechanisms of apoptosis involve the mitochondria, heat shock factors and heat shock proteins, while the extrinsic effects are from Sertoli cells and involve secretion of Fas ligand (FasL) from Sertoli cells and binding to Fas receptors on germ cells that then activate the apoptotic process involving caspases (5). Both the extrinsic and intrinsic pathways also involve the p53 system that triggers proapoptotic events in the mitochondria and translocation to the nuclease and cell cycle stasis and death by binding and regulating DNA expression (5). Heat also results in the disruption of Sertoli cell–Sertoli cell junctional complexes of the BTB that are essential for meiosis to occur in the adluminal space (2,5,12).

While we now know much about the control of spermatogenesis by the BTB and Sertoli cells (2) and that heat induces a variety of molecular changes to the testes (4), the specific sequence of heat stress events remains unclear. Part of the problem is that there are heat effects on both somatic and germ cells. The impact on Sertoli cells to provide control and support of the BTB and the Leydig cells to produce testosterone that have an effect on Sertoli cells is critical

(12,25,26). Another problem is that different models of inducing heat stress are used, for example, 30–120 minutes of 41°C–43°C (or lower) increases in temperature by periodic increases in environmental temperatures, experimental cryptorchid simulation or scrotal insulation.

Early studies found a decrease in testicular weight following heat stress that was associated with evidence of apoptosis upon histological evaluation using histochemical staining or terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (14,15). The initial cells to be affected by heat stress were primary spermatocytes of the first meiotic division and then round spermatids (1). Both short-term and mild heat stress can experience recovery of germ cells, but if the heat stress is continued, then germ cells may not recover suggesting that possibly stem cell spermatogonia can be impacted (1,27).

17.3 INTERACTIONS OF HEAT SHOCK PROTEINS AND HEAT SHOCK FACTORS

Germ cells like somatic cells use heat shock proteins (HSPs) to act as molecular chaperones under normal circumstances and are essential for normal spermatogenesis by ensuring correct assembly and transport of proteins (28). An example of such a HSP is HSP70, the most abundant HSP. Under heat stress, heat shock factors (HSFs), a series of transcription factors, are activated (11). The HSF1 has a dual role to first stabilize and protect somatic cells (heat-resistant cells) but then to trigger apoptosis in germ cells such as primary spermatocytes (heat-sensitive cells) (11). Rather than to preserve germ cells following heat stress, HSF1 appears to trigger germ cell removal, perhaps due to damaged DNA. Widlak and Vydra (11) suggest that this may be due to the overproduction of sperm, and it may be better to just destroy these cells rather than chance the possibility of damaged DNA interfering with fertilization, embryo or fetal development. Even with this removal, we have observed that some sperm produced after heat stress do indeed have damaged DNA and reduced ability to sustain embryo development after fertilization (16,17).

The role of HSFs is complex. In HSF1 knockout mice, among other defects, male mice produce 20% less sperm and more sperm with abnormal head morphology (29–31). In HSF2 knockout mice, there were much fewer sperm produced due to increased apoptosis of primary spermatocytes. However, double knockout mice were completely infertile due to lack of postmeiotic cells, suggesting synergistic roles of HSF1 and HSF2 (32). Other HSFs are expressed in the testis, but roles remain unclear in terms of spermatogenesis. Interestingly, HSF1 and HSF2 regulate expression of some genes that escape postmeiotic sex chromosome repression and allow expression of these genes in round spermatids and particularly in chromatin packaging changes during spermiogenesis (33). The HSF1/HSF2 complex and associated proteins are essential to normal spermatogenesis.

In somatic cells, activation of HSF1 leads to increased HSPs that protect cells, allowing them to survive heat stress. In these somatic cells, the set point for activation of HSF1 is 41°C, but in the testis it is activated at 35°C–38°C (34). Surprisingly, somatic cells of the testes including Leydig and Sertoli cells have profiles of HSF1 activation similar to the general somatic cells of the organism (34). In contrast to somatic cells, germ cell activation of HSF1 does not lead to transcription of antiapoptotic pathway proteins (35–37) and often leads to downregulation of heat shock proteins (38) and upregulation of apoptosis (39,40).

Cells sense heat stress by the activation of a variety of cell signaling mechanisms that can include mitogen-activated protein kinases (MAPK), the extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase and p38, as well as protein kinases A, B and C, Rac1, and CaMKII (5,41). Many of these pathways are initiated by changes in lipids at the level of the plasma membrane (41). All of these signaling mechanisms can impact HSF1, but responses vary by temperature exposure. It is reasonable then to expect that responses to heat stress of cryptorchidism or mild heat stress (37°C–38°C) will be different than responses to acute heat stress at 41°C–43°C. This correlates well with the rapid testis response to 41°C–43°C in which the heat-sensing factors MAPK and p38 were shown to be activated within 6 hours of heat stress and then triggered a reduction in the components of the BTB, inhibiting further meiosis (12). Specific evidence of apoptosis has also been reported (24,42). Even earlier, 0.5–2 hours after heat stress, redistribution of apoptotic regulators is occurring (10,43,44). Recently, acute heat stress has also been shown to change the expression of a series of miRNA and protein acetylation, which have a variety of downstream effects (42,45). In contrast to acute heat stress, longer-term and lower temperatures of cryptorchidism, scrotal insulation or mild environmental temperatures produce a much slower response. Apoptosis increases over 2–4 days following mild heat exposure but uses similar pathways (1,18,21,46–48).

While HSF1 is stable following heat stress, HSF2 degrades rapidly, and its degradation increases with temperature (49) leading to fewer HSF1/HSF2 complexes at higher temperatures such as 41°C (19). As the complexes decrease, HSF1 is released to bind to regulatory components of DNA that promote apoptosis in spermatocytes (11). Thus, at 43°C, a rapid decrease in HSF2 leads to free HSF1 that triggers quick apoptosis responses.

In somatic cells, HSF1 again triggers cell survival, but in spermatocytes and spermatids, it causes proapoptotic events and initiates capase-3 dependent apoptosis (50). Thus, HSF1 has been proposed to be a gatekeeper, and its activation in germ cells triggers the removal of damaged cells rather than their repair or survival (4,11,36).

17.4 EFFECTS OF MODERATE HEAT STRESS

Because moderate heat stress near body temperature (37°C–38°C) for multiple days to weeks occurs at a much slower pace than acute heat stress, more events may come

into play to trigger DNA damage (51). It may be this slow response is more important to understand, as this is what would occur due to daily increases in environmental temperature changes or lifestyle results associated with obesity (4). For example, let's consider reactive oxygen species (ROS) generation. During heat stress, there is an increase in ROS production by the testis (52–54), and this can trigger apoptosis (55,56). While the testis has naturally occurring antioxidants to combat ROS, the effect of heat stress is predominately an increase in ROS (20,57) but has sometimes been associated with decreases in naturally occurring antioxidants (20). Mitigation of moderate heat stress has thus been attempted by increasing antioxidants in the testis (20,52) with often very good results due to blocking increases in ROS and downstream events of apoptosis. However, in addition to direct increases in ROS, there are also downstream events of DNA damage, or the DNA damage might be due to the activation of other pathways (5,20). If examining heat stress for mechanisms of potential contraception, there is no need to repair ROS damage. For livestock, we are interested in preventing heat stress damage to spermatogenesis and production of mature sperm. While treatment with antioxidants reduces impacts of heat stress and increases the speed of recovery (20), it is not clear this should be an approach used. Do we wish to save some sperm that already have damaged DNA and therefore reduced fertility? Antioxidants may be of greater benefit when used under normal environmental conditions to increase sperm production.

Moderate heat stress has also been shown to cause an increase in Leydig cell numbers and a decrease in testosterone (20). The role of testosterone in heat stress has been fraught with conflicting reports (26,58–60). The increase in Leydig cell numbers in heat stress may be due to the prosurvival activation of HSF1 as seen in other somatic cells (34). High heat exposure led to Leydig cell hyperplasia and decreased testosterone production due to effects on cyclins and the steroidogenic enzymes CYP17 and Star (26), which could be mediated by HSF1 activation. The exact mechanisms impacting Leydig cells under moderate heat stress remain unclear and require measurement of intratesticular testosterone to be sure. Heat stress also results in a decrease of the androgen receptor (AR) activation in Sertoli cells leading to disruption of the BTB (61). Part of the effect of heat stress on the AR of Sertoli cells is the upregulation of HSP70 likely via upregulation of HSF1 (18). The high amounts of HSP70 interact with AR and can inhibit testosterone binding. Surprisingly, supplementation of moderate heat stressed mice with antioxidants prevented Leydig cell hyperplasia, decreases in testosterone and other apoptotic changes to spermatocytes, suggesting a powerful role of ROS in this process (20). Despite the previous conflicting response of heat stress on testosterone production and circulating levels, we now conclude that heat stress does lead to decreased testosterone at least within the testis. Further, the effect of heat stress on the AR only enhances this effect leading to disruption of the Sertoli cell support of spermatogenesis. The end result of decreased testosterone support for spermatogenesis is activation of apoptosis by some of the same mechanisms as activated by HSF1 in other aspects of heat stress (5).

During moderate heat stress such as scrotal insulation, although apoptosis occurs, not all the damaged sperm are removed. There are still motile sperm ejaculated that have damaged DNA and fail to support proper embryo development after fertilization (16,17). For example, ejaculated sperm following scrotal insulation express increased FasL, DNA damage via TUNEL assay, increased mitochondrial damage suggestive of increased ROS production and failure to support embryo development *in vitro* following *in vitro* fertilization; all in agreement with sperm DNA damage (16,17). A role of autophagy remains unclear (4). Further examination of such animal models and how sperm escape apoptotic removal could provide insight into why males differ in fertility even without sperm motility or morphological defects.

17.5 DISCUSSION AND FUTURE DIRECTIONS

Spermatogenesis is clearly complex, and understanding the molecular mechanisms has been difficult due to the research goals of either development of contraceptives or improved sperm production. A unified idea of mechanisms is now coming to light by the utilization of heat stress models. Both HSF1 and HSF2 are needed for normal spermatogenesis, and in particular, for meiosis and nuclear changes associated with spermiogenesis. Upon heat stress, a variety of membrane-bound sensors upregulate cell signaling molecules that activate HSF1 and HSF2 leading to increased HSF1 activity in spermatocytes and early spermatids. Release of HSPs, in particular, HSP70 and its transcription, lead to germ cell apoptosis via intrinsic pathways. In Leydig and Sertoli cells, HSF1 activation initiates prosurvival pathways resulting in increased Leydig cell numbers but with decreases in testosterone production. In Sertoli cells, there is downregulation of AR likely due to upregulation of HSF1 and HSP70. The negative effects of decreased androgen response lead to disruption of the BTB and decreased support of meiosis by Sertoli cells. Sertoli cells also secrete FasL that triggers extrinsic apoptosis of germ cells.

Following the initial events of heat stress, one of the downstream consequences is the increase in ROS due either to increased production or to decreased antioxidants. The location is within spermatocytes and spermatids in the testis, which is different than what most andrologists are concerned with—production of ROS in ejaculated sperm. The increased ROS will lead to DNA damage and can be reduced by adding antioxidants, but the approach may be questionable since DNA damage can occur in apoptosis due to other pathways and events. Should we save sperm that perhaps still have DNA damage by other pathways? The production of ROS is just one of many pathways activated via heat stress to trigger apoptosis that should also be considered (4,5,11,42,45,51).

Understanding how HSF1 and HSF2 respond to temperatures in the testis has clarified both speed and downstream

events of different heat stress animal and cellular models. We still need to bring together researchers examining contraception action and maximizing germ cell production/survival to understand how molecular models and events affect spermatogenesis.

17.6 CONCLUSION

Using heat stress animal models has led to a better understanding of the mechanism and events within spermatogenesis. A unified concept of how heat impacts the testis has also brought together divergent areas of research dealing with contraception and improvement in semen quality from rodents, nonhuman primates, primates and livestock species. Previous research has been hard to reconcile in the past, as the research has focused on various aspects of spermatogenesis. Now the total efforts can be used to address the various end goals of the different types of research.

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