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Heat stress and stallion fertility

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Abstract

The threat posed by increased surface temperatures worldwide has attracted the attention of researchers to the reaction of animals to heat stress. Spermatogenesis in animals such as stallions is a temperature-dependent process, ideally occurring at temperatures slightly below the core body temperature. Thus, proper thermoregulation is essential, especially because stallion spermatogenesis and the resulting spermatozoa are negatively affected by increased testicular temperature. Consequently, the failure of thermoregulation resulting in heat stress may diminish sperm quality and increase the likelihood of stallion infertility. In this review, we emphasize upon the impact of heat stress on spermatogenesis and the somatic and germ cells and describe the subsequent testicular alterations. In addition, we explore the functions and molecular responses of heat shock proteins, including HSP60, HSP70, HSP90, and HSP105, in heat-induced stress conditions. Finally, we discuss the use of various therapies to alleviate heat stress–induced reproductive harm by modulating distinct signaling pathways.

Keywords: heat stress, fertility, testicular cells, heat shock proteins, spermatogenesis

Introduction

The current rise in global temperatures is concerning for the horse industry, particularly the stud market, as hot and humid conditions can negatively influence stallion (*Equus caballus*) fertility. Spermatogenesis is a temperature-dependent process that optimally functions at 2℃–4℃ below the body temperature (at 35℃) (1). Testicular hyperthermia due to inefficient scrotal thermoregulation may cause genital heat stress and, consequently, detrimental effects on spermatogenesis (2). The fertility index and per cycle conception rates of stallions are low compared with those of other animals because, unlike for other domestic species, the selection of stallions for breeding depends primarily on racetrack performance record and conformation rather than reproductive soundness and heritable traits (3). The reduced fertility or complete sterility experienced by most stallions are consequences of different environmental factors, including incomplete testicular descent, malnutrition, hormonal imbalances, chemicals, drugs, and elevated scrotal temperature (4). When testicular temperature increases because of fever, high ambient temperature, or inflammation, the metabolism increases at a faster rate than the blood flow, consequently rendering the testes hypoxic. Horses are mostly kept for racing purposes and a considerably high scrotal temperature has been observed during exercise, which may cause testicular insult and disrupt spermatogenesis (4).

Heat shock proteins (HSPs) are naturally expressed in cells (5). Their expression can be stimulated to protect the cells in response to stress conditions induced by cellular injury, environmental changes, and high temperatures.

HSPs are expressed in males and females of numerous species and play a crucial role in the physiology of reproduction (6). The expression of different HSPs is highly conserved in different parts of the stallion sperm (7), thereby indicating the role of HSP not only in germ cell development and sperm motility (8) but also in mitochondrial protein folding, gamete interaction, and signaling associated with capacitation (9).

High temperatures during summer may negatively affect stallion fertility (10), as demonstrated by changes in hormone secretion and semen quality (11). These effects may be influenced by the age of the stallion and mediated by the combined effects of obesity and oxidative stress. Management measures, such as using cooling systems, supplementing diets with antioxidants (12), and scheduling outdoor activities during the cooler parts of the day, should be considered for mitigating the negative effects of heat stress on stallion fertility.

Effects of heat stress on stallion fertility

Normal spermatogenesis requires maintaining the testes at a temperature lower than that of the body. When the testicular temperature rises owing to fever, inflammation, or high ambient temperature, testes become hypoxic as the metabolism increases more rapidly than the blood flow (13). Hypoxia can cause cell apoptosis, consequently triggering testicular degeneration (13). Extensive activity substantially increases the core body temperature of horses to values >41℃ (14). The most common testicular thermoregulation methods in animals involve scrotal sweat glands, scrotal muscle relaxation, heat loss from the scrotal surface, and the arteriovenous countercurrent heat exchange mechanism at the pampiniform plexus (15). Optimum stallion fertility requires appropriate thermoregulation. A study conducted by Carlos et al. reported that rectal and body surface temperatures following sun exposure were increased, whereas the scrotal surface temperature (SST) remained unchanged owing to efficient thermoregulation in stallions (16). The inability to thermoregulate scrotal temperatures causes testicular hyperthermia and genital heat stress, which are harmful to spermatogenesis and result in low-quality spermatozoa (2). Furthermore, numerous testicular insults can alter the chromatin structure of spermatozoa, inducing spermatozoal DNA denaturation (17). Love and Kenney reported that stallion spermatozoa with denatured DNA contain fewer disulfide bonds and exhibit increased DNA sensitivity to denaturation (18). They further observed that the vulnerability of spermatozoal DNA to denaturation depended on the spermatogenic cell stage at the time of heat shock (18). Reduced fertility has been linked to a higher rate of spermatozoal DNA denaturation in bulls (19) and humans (20), probably because the extent of intramolecular and intermolecular disulfide bonds of the protamine molecule plays a crucial role in the decondensation process that occurs immediately after fertilization. Additionally, the number of disulfide bonds in the spermatozoal nucleus affects the time of decondensation (21),

and this timing probably depends on the type of protamine present (22). Thus, the male and female genomes need to decondense to unite and form a zygote (23).

Horses are primarily housed for racing purposes. High intensity exercise considerably increases the core body temperature to values of >41℃ (24). Therefore, ambient airflow is critical during exercise to control SST and avoid potential damage to spermatogenic cells due to heat stress in stallion (25). Previous studies have reported that stallions experienced the exercise either through riding or treadmill wearing the suspensory have a significant influence on SST (26). The SST was 2℃ higher in the stallions wearing a suspensory than in those without a suspensory (26). Reportedly, a small but recurrent rise in subcutaneous scrotal temperature of 1.4℃–2.0℃ considerably reduced fertility in ram (27). The superficial testicular veins rapidly respond to variations in SST, and these changes influence the temperature of testicular arterial flow through heat exchange in the pampiniform plexus (28). Therefore, it is recommended to remove the suspensory immediately after exercising to maintain ambient airflow and prevent any heat stress to the spermatogenic cells. Thus, reduced sperm quality and maturation may be associated with elevated testicular temperatures. Indeed, the morphological defects and sperm head alterations positively correlate with SST in stallions (16).

Effects of heat stress on the different testicular cell types in stallions

Different testicular cell types respond differently to heat stress in terms of sensitivity, response, and physiological and pathological changes. However, spermatogenic cells are more susceptible to heat stress–induced damage than other cells as they undergo cell division frequently and lack superoxide dismutase (29). Study revealed that spermatocytes and mature sperm cells are sensitive to temperature and that zygotene and pachytene spermatocytes and early round spermatozoa are the cells most susceptible to heat damage in rats (30). Recently, our research team reported increased apoptosis of testicular cells, including somatic and spermatogonial stem cells, under heat stress in cell culture conditions compared with that at normal temperatures (in press). Another study involving porcine testes reported significantly higher Bcl-2 protein and *mRNA* expression levels following heat treatment than those in controls, indicating that apoptotic signals were stimulated under heat stress conditions and that spermatocytes and spermatids comprised the most affected cell types (31). Heat negatively impacts the integrity of spermatocytes and breaks double-stranded DNA; thus, DNA damage constitutes an additional cause of heat stress–induced apoptosis during spermatogenesis (32). Furthermore, heat stress may induce aberrant sex chromosome segregation during meiosis, producing unpaired Y chromosomes and consequently triggering spermatocyte apoptosis. DNA repair during spermatogenesis in developing germ cells is important for meiotic recombination (33). Recently, the role of deleted in azoospermia-like (DAZL), present in the different germ cell

types (differentiated spermatogonia and primary spermatocytes) of stallion testes (34), in germ cell fate has been discovered. Endogenous DAZL is involved in generating stress particles, including 40S ribosomal subunits, RNA-binding proteins, translation initiation factors, and polyadenylated mRNAs, that react to different environmental stresses and are implicated in spermatocytes survival (35). Moreover, thermal damage affects spermatogonial stem cells (SSCs), although SSCs mostly recover independently and rarely undergo heat stress–induced apoptosis. A previous study revealed that heat treatment in SSCs changed the self-renewal, protein localization, and protein folding, causing cell cycle arrest, but did not substantially alter the expression levels of apoptosis-related genes (36). Undifferentiated transcription factor 1 (UTF-1) is reportedly expressed in undifferentiated spermatogonia (34) and DDX4/MVH (VASA) is expressed in the cytoplasm of spermatogonia, primary spermatocytes, and round spermatid in stallions. VASA immunolabeling intensity is substantially greater in pachytene spermatocytes than in spermatogonia and round spermatids (37). Recently, our laboratory (data not published) detected no significant difference in the UTF-1 *mRNA* expression levels between normal and cryptorchism stallion testes, whereas the VASA *mRNA* expression levels were significantly lower in cryptorchidism testes than in normal testes. These results further strengthen the hypothesis that heat stress does not exert a long-lasting effect on SSCs and that pachytene and primary spermatocytes are the cell types most affected by heat stress.

Leydig cells, located within the Leydig portions of seminiferous tubules release testosterone, which is controlled by gonadotropin-releasing hormone released from the hypothalamus. Leydig cells produce >95% testosterone in mammals (38). There is a significant association between the testosterone levels of stallions and their fertility. Inoue et al. reported that azoospermic stallions exhibit considerably lower testosterone levels than normal adult stallions (39). Furthermore, in another study, the serum testosterone levels in stallions found significantly different in hot summer conditions (11). Lipids are precursors of androgen synthesis (40). When mouse Leydig cells are exposed to heat stress, there is an increase in lipid accumulation which suggests that heat-shocked cells experience a disruption in testosterone production. In rats induced with scrotal hyperthermia, the number of testosterone-positive Leydig cells was considerably lower than that in the control group, as demonstrated via immunohistochemistry (41). Studies have revealed that, following heat treatment, the expression levels of the androgen receptor and junction-associated proteins, such as occludin and zonula occludens-1 (ZO-1), is significantly reduced in SSCs (42).

Sertoli cells are essential components of the brain–testis barrier (BTB) and are the principal supporting cells of the spermatogenic epithelium, supplying the nutrients and support required for spermatogenic cell development (43). The BTB is essential for spermatogenesis and plays a crucial role in testicular physiology and pathology (44). Increase in testicular temperature disrupts the function and shape of Sertoli cells, leading to infertility and

germ cell death. Recently, our laboratory reported significantly high apoptosis rate in the testicular cells of stallions subjected to heat stress in vitro (in press). Alterations in various BTB-associated proteins due to scrotal heat stress induce ultrastructural BTB damage and reversible spermatogonial cell dedifferentiation (45). Cai et al. reported that, following 48 h of brief heat treatment (30 min) at 43℃, the protein and *mRNA* expression levels of the tight junction molecules ZO-1 and occludin in the BTB of mice drastically decreased, resulting in loose tissue and high permeability (46). Further, they demonstrated that the expression levels of two proteins, Wilms' Tumor 1 protein and transferrin, which are markers of Sertoli differentiation and secretion function, were reduced in Sertoli cells. Additionally, the organization of microtubule (α- and ß-tubulin) and microfilament (f-actin) networks was lost, suggesting that cytoskeletal changes occur under thermal stimulation (42). Moreover, thermal stimulation alters the expression levels of the BTB components, including ZO-1, connexin 43, claudin 1, claudin 5, and vimentin, as well as the expression levels of *mRNA*s encoding the inflammatory cytokines interleukin (IL)-1α, IL-1 β, and IL-6 (47). Thus, heat treatment induces the breakdown of cell junctions (42) and disrupt the spermatogenesis.

Functions of heat shock proteins in stallion spermatogenesis

Heat shock proteins (HSPs) were discovered in cells exposed to high temperatures. They constitute a very intricate and well-preserved cellular defense system and play a crucial role in maintaining cell viability in unfavorable environmental circumstances. HSPs perform two key tasks. First, they function as molecular chaperones under physiological circumstances, facilitating the transport and folding of other intracellular proteins alongside assembling proteins into oligomeric structures in certain situations. However, HSPs do not constitute the final protein structure. Furthermore, HSPs play vital functions in intracellular trafficking, preserving proteins in their inactive states, and preventing protein breakdown (48). Second, HSPs are selectively expressed in response to various stressors, including temperature variation, inflammation, bacterial and viral infections, heavy metals, and free oxygen radicals (49). The term "heat shock response" denotes the stress-induced activation of heat shock genes and is widely observed in clinical conditions, such as circulatory and hemorrhagic shock and ischemia. Cellular stress alters the tertiary structure of proteins, exerting negative consequences on cellular metabolism. However, a "stress tolerance" phenomenon induces HSP expression and protects cells from insults as HSPs interact with intracellular polypeptides to prevent improper protein assembling or denaturation (6). The molecules in the heterogeneous family of HSPs are often categorized based on their molecular weight as HSP60, HSP70, HSP90, or HSP105. Numerous molecular complexes with receptor functions have been detected in the sperm tail and midpiece of spermatocytes. HSPs may be components of receptors that are either directly engaged in

controlling motility or indirectly involved in promoting the folding of the hormone-binding domain of receptors to a high-affinity hormone-binding conformation (7).

HSP60 immunoreactivity was detected in the midpiece of spermatocytes of various animals, including stallions (7). HSP60 facilitates the ATP-dependent proteolytic destruction of misfolded or denatured proteins by participating in mitochondrial protein folding (50). Owing to the significant resemblance between its bacterial and human amino acid sequences, HSP60 is well recognized as a key autoantigen in various autoimmune disorders and pathogenic infections (51). In stallions, HSP60 expression has been correlated with environmental temperature and semen quality and exposure of high temperature leads to low semen quality and decreased male fertility (52). Albrizio et al. reported that the highest HSP60 expression levels were recorded in stallion spermatozoa during the months with the highest total number of hot days (9). However, the HSP60 expression levels were the lowest in December. Reportedly, photoperiod influences horse reproduction (53), and fall and winter anestrus are characterized by short daylengths. Therefore, we hypothesize that photoperiod affects HSP expression levels. HSP60 is involved in regulating apoptosis of cells, including Sertoli cells and spermatogonia. In a study involving monkeys, an increase in apoptotic spermatocytes and round spermatids and HSP60 expression levels was observed on days 3, 8, and 30 following a temporary rise in testicular temperature (43℃ once/day for 2 consecutive days) (54). The antiapoptotic effects of HSP60 and HSP10 have been demonstrated in several cell types. Additionally, the expression of these two proteins may be upregulated in response to cellular stress (55). Shan et al. overexpressed HSP60 and HSP10 and discovered that both HSPs independently influence the post-translational modifications of the members of the Bcl-2 protein family (56). Furthermore, HSP60 overexpression was linked with greater Bax suppression, more pronounced Caspase 3 inhibition and improved Bcl-xl induction along with downregulated Bad in doxorubicin-treated cells (56). These findings indicate that HSP60 induces antiapoptotic properties in cells, including somatic and germ cells. Recent research has shown that HSP60, in addition to providing protection against stress, is important for sperm's ability to fertilize eggs, and it has been postulated that the immunological response of these HSPs may play a role in male infertility (57).

HSP70 is one of the most common chaperone proteins. According to previous research, HSP70 plays a major role in the biological processes including protein synthesis and energy metabolic processes for sperm motility (58). HSP70 is essential for spermatogenesis as it protects cells from oxidative stress and apoptosis (59) and plays a role in sperm maturation and sperm–egg recognition (60). It is expressed in mature sperm and male germ cells during spermatogenesis in mice (61), humans (62), bulls (63), boars (64), and stallions (7). The localization of HSP70 is highly conserved among species and between fresh ejaculate and after capacitation and acrosomal reaction. Volpe et al. reported HSP70 localization in boar sperm on the equatorial segment in a triangular region,

whereas the fluorescent signal shifted to the subequatorial band following the capacitation and acrosomal reaction (AR). Only ~50% freshly ejaculated spermatozoa of a stallion demonstrated a positive signal for HSP70 in a thick postacrosomal band; conversely, this signal was visible in ~85% spermatozoa following the AR stimulation. The immunolocalization of HSP70 in the subequatorial region of stallion sperm and the increase in this localization following capacitation and AR indicates the significant role of HSP70 in sperm maturation (7). According to a recent study conducted by Albrizio et al., HSP70 expression in stallion semen is directly proportionate to the duration of daylight (9). Indeed, the expression levels of HSP70 increases during the breeding season, decreases during the fall transition and winter season, and finally increases again during the spring transition. In the same study, Albrizio et al. also investigated the positive correlation between environmental variables and equine semen quality as well as sperm kinetics, including total motility, progressively motile sperm percentage, and average path velocity (9). Similar research conducted in goats revealed that the HSP70 *mRNA* levels were higher during the peak summer season than during the peak winter season. These results indicate that HSP70 expression is directly correlated with semen quality in stallions (65). Conversely, Erata et al. reported that alongside increase in DNA damage, the HSP70 expression levels increase in the sperm of infertile men (66). Furthermore, in vitro experiments revealed that treatment with an anti-HSP70 antibody decreases fertilization rate in a dose-dependent manner, indicating that HSP70 is important for the interaction between sperm and oocytes (64). Further research on HSP70 expression will be beneficial for comprehending its precise function and may contribute to improving assisted reproductive technologies to tackle male infertility.

HSP90 is a highly abundant and ubiquitous chaperone protein that plays a crucial role in cell survival, cell cycle regulation, and hormone and other signaling pathways. Volpe et al. first reported HSP90 expression in stallion spermatozoa. Although HSP90 immunoreactivity sometimes appears in the neck or midpiece, it is mostly detected throughout the tail of spermatozoa. Capacitation and AR do not appreciably change the HSP90 localization of stallion spermatozoa. The tail location of HSP90 in the mature fresh semen of stallions may be crucial for the signaling processes involved in capacitation, and thus, HSP90 may impact the fertilization ability of spermatozoa. Nitric oxide (NO) is a proven essential component of several signaling pathways regulated via cAMP and protein kinase A (PKA) (67), which induce tyrosine phosphorylation (68). HSP90 efficiently activates NO synthase (NOS), thereby stimulating NO synthesis (69). PKA in the fibrous sheath of the sperm flagellum has been related to tail-associated tyrosine phosphorylation that occurs only in the midpiece and principal tail regions of the capacitated spermatozoa of stallions (70). HPS90 possibly significantly influences sperm motility. HSP90 may interact with its protein partners engaged in signaling cascades or with the hormone-binding domains of receptors located on the sperm tail to modulate the motility of subcellular structures, such as the axoneme or thick outer

longitudinal fibers. Both treatment with the HSP90-specific inhibitor geldanamycin (8) and decreased HSP90 levels during cooling or after cryopreservation reportedly reduce porcine sperm motility (71). HSP90 plays a role in maintaining the integrity of mitochondria in sperm cells. It is a chaperone protein that helps fold and stabilize other proteins in the cell. Reportedly, HSP90 interacts with several proteins involved in mitochondrial function in sperm. One study reported that HSP90 is required for the proper assembly and stability of the mitochondria-associated membrane (MAM), a structural and functional unit that connects the endoplasmic reticulum (ER) and mitochondria in cells. The MAM is imperative for maintaining mitochondrial integrity and regulating calcium ion flow between the ER and mitochondria. In sperm cells, the MAM maintains the structural integrity of the mitochondria and ensures that the mitochondria can provide the energy required for sperm motility. The dysregulation of HSP90 or the MAM causes defects in mitochondrial functions and impairs sperm motility (72). Additionally, HSP90 *mRNA* levels increase in migrating primordial germ cells (PGCs), and reducing HSP90 activity delays cell cycle progression, in turn causing defects and compromising the arrival of PGCs to their destination, i.e., the area where the gonad develops (73). Because cells spend a longer time in the S/G2/M stages during low HSP90 activity conditions compared with high HSP90 activity conditions, it might decrease cell displacement and compromise PGC polarity, thereby preventing cells from rapidly reacting to dynamic changes (73). High HSP90 levels in the presence of testosterone boost DNA methylation in testicular cells. Testosterone-treated rats with varicoceles exhibit higher HSP90 expression levels in spermatogonia, spermatocytes, round spermatids, and Sertoli cells than untreated rats with varicoceles. In line with this data, HSP90 has been involved in the protection and repair of DNA in germ cells and spermatozoa level (74) by promoting protein folding, preventing protein aggregation (75), tightening and condensing chromatic structure, and facilitating chromatin remodeling (76). Obesity negatively impacts spermatogenesis, sperm morphology, and sperm count. Increasing HSP90 expression levels in response to an obesity-induced stress state preserves the nucleotide and protein contents and cellularity of the testes, ultimately preserving male fertility. A recent study reported high HSP90 expression levels in pachytene spermatocytes and round and elongated spermatids in obese rats (77). In the same study, researchers also reported a relatively high proportion of HSP90-positive cells among the cells in the seminiferous tubules and high HSP90 expression levels in the total germ and Sertoli cells (77).

The 105-kDa HSP, also known as HSP105 alpha, is a member of the high molecular mass HSP family. Although it is constitutively expressed, it may be activated in different mammalian cells, including germ cells, via various stressors (78). Depending on the cell type and the nature of the disturbance, HSP105 alpha may protect neuronal cells from apoptosis (79) or promote the death of embryonic cells in response to stress (80). Zhang et al. investigated changes in HSP105 expression during spermatogenic recovery before and after heat exposure of

monkey testes (54). They found a marked decrease in the number of spermatids and expression levels of HSP105 from days 3 to 30 following heat treatment. Additionally, once the cells had recovered from the thermal stress, the HSP105 expression levels returned to the pretreatment levels. Based on these changes due to heat stress, we hypothesize that either HSP105 expression levels reduced because of heat-induced germ cell death or the germ cells underwent apoptosis because of the diminished ability of HSP105 to protect spermatids (54). At high temperatures, nuclear chromatin is condensed in the scrotum, further activating p53 and inducing its translocation toward the nucleoplasm where it induces cell cycle arrest or cell death (81). Notably, elevated scrotal temperatures may promote HSP105 binding to p53, thereby retaining p53 in the cytoplasm and preventing it from exerting its nuclear functions. Therefore, this HSP105-dependent p53 stability may stop p53 from initiating apoptosis (82). Overall, we infer that HSP105 is involves in the heat-induced death of germ cells.

Possible supplementation and management measures for preventing heat stress–induced stallion infertility

Combating heat stress in high-temperature climatic conditions is challenging. Research involving the effects of different antioxidants, neuroendocrine hormones, and traditional herbs in laboratory animals and other livestock species subjected to heat stress has considerably progressed. However, such studies are scarce in stallions, and comprehensive studies are warranted to confirm the ability of these remedies to maintain optimum fertility in heat stress conditions.

Antioxidant compounds, such as vitamin C, reportedly alleviate oxidative stress and reduce the risk of cellular damage. Particularly, vitamin C is an effective water-soluble antioxidant as it can neutralize reactive oxygen species (ROS) in the water phase and prevent lipid peroxidation (83). In vitro studies have demonstrated that prophylactic treatment with vitamin C partially protects Sertoli cells from short-term heat stress in mice. Supplementation with 20 or 50 μg/ml vitamin C considerably increases the viability of TM4 Sertoli cells under heat stress conditions. Additionally, pretreatment with vitamin C reduces oxidative stress, increases HSP expression levels, and prevents microtubule aggregation in Sertoli cells. These effects potentially help mitigate Sertoli cell apoptosis due to heat stress and restore the protective function of the BTB toward germ cells (84).

Melatonin, a hormone produced by the pineal gland (85) and also synthesized in the testes (86), exhibits potent antioxidant properties. It activates various antioxidant enzymes, scavenges free radicals, and protects against inflammation in the testes (87). In mice, melatonin injection (20 mg/kg per day) before hyperthermia induction alleviates reproductive damage by inhibiting the apoptotic JNK and p38 MAPK signaling pathways, thereby reducing apoptosis and oxidative stress. Melatonin treatment following heat stress improves the histological indices in the seminiferous epithelium, germ cells, and testes in mice and strengthens the integrity of Sertoli cells

tight junctions (88). These findings suggest the potential of melatonin for treating subfertility or infertility due to various testicular hyperthermia factors.

Additionally, traditional medicines can used to alleviate heat stress–induced reproductive harm. Korean red ginseng (KRG) is a traditional herb commonly used to increase libido and improve male fertility (89). A study involving rats reported that the administration of KRG extracts during long-term heat stress upregulates the protein and *mRNA* levels of antioxidant enzymes (glutathione peroxidase 4, glutathione S-transferase μ5, and peroxiredoxin 4) in the testes. The administration of KRG at a dose of 100 mg/kg/day counteracts the changes in these heat stress–induced antioxidant indices in the testes, thereby improving the resistance of the testes to oxidative stress due to heat and enhancing the physiological functions of the testes. Therefore, KRG provides a conducive environment for spermatogenesis (90). These findings suggest that KRG is a promising therapeutic agent against hyperthermia-induced male infertility. Previous study has demonstrated that baicalin, a flavonoid present in *Scutellaria baicalensis*, exhibits a range of pharmacological activities (91). These include the ability to reduce cellular stress and apoptosis (92). Pretreatment with baicalin also reduces the expression of P-JNK, FAS, FASL, caspase-9, caspase-3, and APAF-1, suggesting that baicalin inhibits the FAS/FASL apoptosis pathway in Sertoli cells of heat-stressed mice (93). The edible plant *Angelica keiskei* (*Ashitaba keiskei*), native to Japan, contains the active ingredients xanthoangelol and 4-hydroxyderricin, which constitute the primary polyphenol compounds of the plant and possess antiobesity, hypotensive, and antidiabetic activities alongside other beneficial properties (94). Supplementation with Ashitaba powder (AP) prevents the reduction in HSPa11 and HSPa2 expression levels due to short-term heat stress in the testicular cells of mice. The HSPa11 and HSPa2 expression levels in the testes are crucial for fertility. Furthermore, AP may reduce heat stress–induced ROS production by enhancing the glutathione synthase and heme oxygenase-1 expression levels. The AP-mediated increase in the activities of HSPs and antioxidant enzymes mitigate the toxic effects of heat stress, including ROS generation (95). Thus, AP supplementation may help prevent heat stress–induced male infertility. In another study, in seminiferous tubules, quercetin supplementation decreased the rate of apoptosis of germ cells while maintaining the interstitial stroma, seminiferous tubule architecture, germinal, and Sertoli cells under heat stress conditions (96). Study showed, in TM3 Leydig cells exposed to heat stress, zinc supplementation was demonstrated to be a possible protective factor against apoptosis and decreased testosterone synthesis (97). In conclusion, supplementation with the abovementioned antistress remedies and implementing management measures should enable the minimization of the risk of heat stress–induced infertility in stallions. Numerous management measures can be enforced, such as 1) providing adequate shade to horses, either in the stable or on the track, to reduce their body temperature; 2) removing the suspensory immediately after exercise to avoid potential heat-induced harm to

spermatogenic cells; 3) ensuring proper hydration by providing horses with an ample supply of water, particularly during hot weather conditions, to help maintain hydration levels and prevent heat stress; 4) utilizing cooling techniques, such as hosing the horses down with cool water or applying ice packs to their necks and chests to lower their body temperature; 5) adjusting the workload during hot weather conditions by reducing the intensity and duration of the exercise to prevent heat stress; 6) providing adequate transportation to the horses in well-ventilated trailers with access to water; and 7) closely monitoring the behavior and appearance of horses and being vigilant for signs of heat stress, such as excessive sweating, increased respiratory rate, and decreased appetite. By implementing these strategies, it is possible to help prevent heat stress and mitigate its negative effects on stallion fertility.

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No potential conflict of interest relevant to this article was reported.

Author's Contributions

M.Y.: Conceptualization, Resources, review & editing.

M.S.: Writing - original draft, validation, and visualization.

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