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Heat stress induced by testicular insulation for 24 or 48 hours rapidly impairs epididymal sperm quality and reduces spermatogenesis in rams

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Abstract

Mammalian testes must be 3 - 5 ºC below body core temperature to produce morphologically normal sperm. The objective was to investigate impacts of heat stress (HS) induced by scrotal insulation on epididymal sperm and temporal aspects of HS on spermatogenesis. We hypothesized that: 1) increased testicular temperature impairs sperm in the epididymis; and 2) spermatids are severely impacted by HS exposure. Testicular HS was induced by scrotal insulation for 24 or 48 h in 20 reproductively sound adult rams, with 5 similar rams designated controls (not insulated). Rams were castrated at 24 h, 48 h, 7 d, or 14 d after the start of insulation (whereas control rams were randomly castrated). Insulation increased scrotal surface temperature by ~5 ºC. There were marked decreases ($P<0.01$) in sperm motility, progressive motility and kinetics starting at 24 h and sustained throughout the study. Percentage of epididymal sperm with normal morphology first decreased at 24 h ($P<0.01$) with subsequent decreases at 48 h ($P<0.01$) and 7 d ($P<0.01$); thereafter, morphology remained stable ($P>0.05$). At 14 d, there were decreases in testicular weight ($P<0.05$) and seminiferous tubule diameter (STD) ($P<0.001$) when compared to all other groups. Regarding seminiferous tubule integrity (Johnsen's score), a first decrease occurred at 24 h ($P<0.05$) followed by a more intense decrease at 14 d ($P<0.001$). In addition, there was an abrupt decrease ($P<0.05$) in spermatid counts at 24 h that was sustained throughout the study. In conclusion, our hypotheses were supported; testicular HS caused immediate deleterious impacts on epididymal sperm at 24 and 48 h post-insulation as well as developing spermatids at 7 and 14 d, decreasing sperm production and significantly reducing both STD and testicular weight.

Keywords: Testicular hyperthermia, sperm motility, sperm morphology, testis, seminiferous tubule.

Introduction

Testicular temperature must be 3 to 5 °C below core body temperature for proper testicular function and spermatogenesis, with increases in testicular temperature impairing sperm production and reducing sperm quality (Kastelic et al., 1996). Climate change and heat waves highlight the importance of understanding impacts of heat stress (HS) on animal production and reproduction. Heat exposure is often an isolated event, and animals can physiologically compensate with vascular and non-vascular thermoregulatory responses (Rizzoto et al., 2020D). However, HS that challenges thermoregulatory capability can be either acute (Rizzoto et al., 2020B) or chronic (Kastelic et al., 2017).

The pathophysiology underlying the deleterious effects of testicular heat stress (HS) has been documented (Rizzoto et al., 2020A and B). Activation of apoptotic pathways, causing death of spermatogenic cells, is a critical process that reduces sperm number and quality. Although various stages of spermatogenesis may be affected, spermatids and spermatocytes are particularly susceptible to apoptosis post-HS exposure (Rockett et al., 2001).

In rams, spermatogenesis takes ~ 42 d to be completed (Bilaspury and Guraya, 1986); cells that were spermatids and spermatocytes during HS were ejaculated at 14 d post-HS, when sperm quality was at its nadir (Rockett et al., 2001). The main features of impaired sperm quality include severe reductions in motility (Hamilton et al., 2016), a higher percentage of morphologically abnormal sperm (Armengol et al., 2015), reduced sperm output, and lower fertilizing potential (Mieusset et al., 1992; Rizzoto et al., 2020A).

After sperm are released, they are transported through the epididymis (head, body, and tail), and undergo essential maturation changes in both their plasma membrane and metabolic characteristics (Hammerstedt et al., 1979; Inskeep and Hammerstedt, 1982). Importantly, modifications in the epididymal micro-environment can adversely affect sperm maturation, impacting functions both before and after fertilization (Ayaz et al., 2021). Despite the crucial

role of epididymal function in male reproduction, few studies have examined effects of HS on epididymal function in bulls (Alves et al., 2016; Ross and Entwistle, 1979) or rams (Hamilton et al., 2016).

The objective was to investigate impacts of HS (induced via scrotal insulation) on epididymal sperm and temporal aspects of HS on spermatogenesis. We hypothesized that: 1) increased testicular temperature impairs sperm in the epididymis (at 24 and 48 h post-insulation); and 2) spermatids are severely impacted by HS exposure (at 7 and 14 d post-insulation).

Materials and methods

This study was conducted during the breeding season for sheep in the southern hemisphere (May to July). All activities were approved by the local institutional animal care committee (FMVZ – UNESP, Botucatu, SP, Brazil – Permit number 041/2021 - CEUA). Twenty-five crossbred rams (Santa Inês x Dorper), ~ 1 y and weighing 55 kg, with body condition score ≥ 3.5 (1-5 scale; Kenyon et al., 2014) were used. All rams were maintained in stalls in groups of 2 or 3 and fed concentrate and hay, according to the Nutrient Requirements of Small Ruminants (2007), with *ad libitum* access to water. Prior to the study, all rams had undergone a standard breeding soundness evaluation and were deemed acceptable, according to Brazilian College of Animal Reproduction standards (CBRA, 2013).

Rams were randomly assigned to the following experimental groups (n = 5 per group): Control (no scrotal insulation), 24H (24 h of insulation followed by immediate castration), and 48H, 7D and 14D (48 h of insulation followed by castration immediately or 7 or 14 d after the start of insulation, respectively). Disposable baby diapers, covered with medical tape, were used to insulate the scrotum and spermatic cord and induce testicular hyperthermia, similar to a previous study (Rizzoto et al. 2020A). The diapers were held in place by skin sutures into

abdominal skin. Tape was used to hold diapers and keep them dry, but was not tight around the scrotal neck and did not affect blood flow.

Scrotal surface temperature was assessed using thermograms captured immediately before and after removing insulation. These images were obtained with a thermographic camera (Flir Model E53 24º, MSX®, Wilsonville, OR, USA). All assessments were performed by the same person (GR) in the same position, location, and at the same time of day. In addition, rectal temperature was obtained with a clinical thermometer immediately before insulation was placed and immediately after its removal.

Castrations were performed using a semi-open technique (Kersjes et al., 1985). Pre-operatively, rams were given 40,000 IU/kg of penicillin G benzathine IM, 2.2 mg/kg of flunixin meglumine IM and 0.2 mg/kg of acepromazine IV. After 15 min, 2% lidocaine with epinephrine were administered: 5 mL per spermatic cord and 10 mL at the scrotal skin incision site. The scrotal skin was then scrubbed, and the orchiectomy performed by the same 2 veterinarians (AGP and ESR). At 8 h post-surgery, a second dose of flunixin meglumine was administered. Thereafter, the rams were monitored every 24 h using the Unesp-Botucatu Sheep Acute Pain Scale (USAPS) (Silva et al., 2020). Additional doses of flunixin meglumine were given to rams with indications of pain.

After castration, testes were immediately weighed and kept in warm saline (37 °C) for further processing. The tail of the epididymis was isolated and maintained in warm PBS (37 °C), then subjected to retrograde flushing to collect epididymal sperm that were immediately evaluated for motility and kinetics. An aliquot (10 µL) of PBS was placed in a Makler® chamber (SEFI Medical Instruments Ltd., Haifa, Israel), and 5 fields were randomly analyzed by CASA (Computer assisted sperm analysis - IVOS, Version 14, Hamilton-Thorne Bioscience, Beverly, MA, USA), using the following setup: frames/field: 30; minimum contrast: 60 pixels; minimum

size: 6 pixels; linearity: 70%; motile threshold speed: 30 μm/s; threshold for average minimum speed: 40 μm/s; threshold for minimum linear speed: 20 μm/s; head displacement: 90; size of head: 5 pixels; amplification: 1.95; and temperature: 38 ºC). Each sperm sample underwent random analysis of 5 fields to determine total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), progressive velocity (VSL, μm/s), linearity (LIN), and straightness (STR). Additionally, sperm were fixed in 4% formalin and morphology (Barth and Oko,1989) assessed by phase-contrast microscopy at 1000X.

Immediately after testes were collected, ~ 1 cm^3 cubes of testicular parenchyma were fixed in 4% formalin, embedded in paraffin blocks and cut into 5-μm sections using a microtome. Slides were prepared and stained with hematoxylin and eosin. Seminiferous tubule diameter (STD) was assessed as described (Rizzoto et al., 2020A), using microscopy at 1000X magnification, with images captured for digital measurement. Fifty cross sections of tubules were measured using ImageJ® software (NIH, Bethesda, MD, USA) and results were averaged and compared among groups. In addition, the same slides were used for histological scoring based on Johnsen's score, following a decreasing value based on tubule quality, with a 10 given to a round and structurally sound tubule, with all spermatogenic stages and sperm in the lumen and 1 being the lowest grade, with no cells detected within the tubule (Johnsen, 1970).

To assess spermatid concentration, a modification of a previous protocol (Ban et al., 1995) was used. The testicular parenchyma sample was thawed, weighed, place in 1 ml of milliQ H_2O, sonicated for 30 s, with the resulting suspension kept at 4 ºC for 1 h, then diluted in milliQ water 1:15 v/v and stained with 20 μl of eosin nigrosin. From the stained suspension, 10-μl aliquots were added to each side of a Neubauer chamber and a spermatid count performed and normalized by testicular weight, to determine spermatids/mg of testes.

Data were assessed for normality, and upon confirming a normal distribution, comparisons were made using ANOVA, followed by Tukey's test. Statistical significance was

considered at $P < 0.05$. GraphPad Prism®, Version 6.0 software (GraphPad Software Inc., La Jolla, CA, USA), was used for all statistical analyses.

Results

Regardless of its duration (24 or 48 h), scrotal insulation increased scrotal surface temperature by ~5 ºC ($P < 0.05$, Fig. 1A) and decreased sperm quality ($P < 0.001$, Fig. 1A). In contrast, rectal temperature remained unaffected ($P > 0.01$, Fig. 1A), indicating that the responses observed were due solely to the insulation and were not systemic.

Regarding epididymal sperm quality, there were significant decreases in the percentage of sperm with normal morphology (Fig. 1B) and in both TM and PM, starting at 24 h (Fig. 1C). Both TM and PM were markedly lower after treatment in all experimental groups, whereas the percentage of sperm with normal morphology was progressively lower according to duration of insulation or time after insulation (24H > 48H > 7D = 14D; $P < 0.05$). morphological changes were largely due to sperm tail abnormalities, which increased ($P < 0.05$) from 24 h onwards (Fig. 2B). Sperm kinetic end points (VSL, VAP, VCL, STR, and LIN, Fig. 2D) had decrease patterns similar to those for TM and PM ($P < 0.05$, Fig. 2C), with no difference for VCL, ALH or BCF at any time point ($P>0.05$, data not shown).

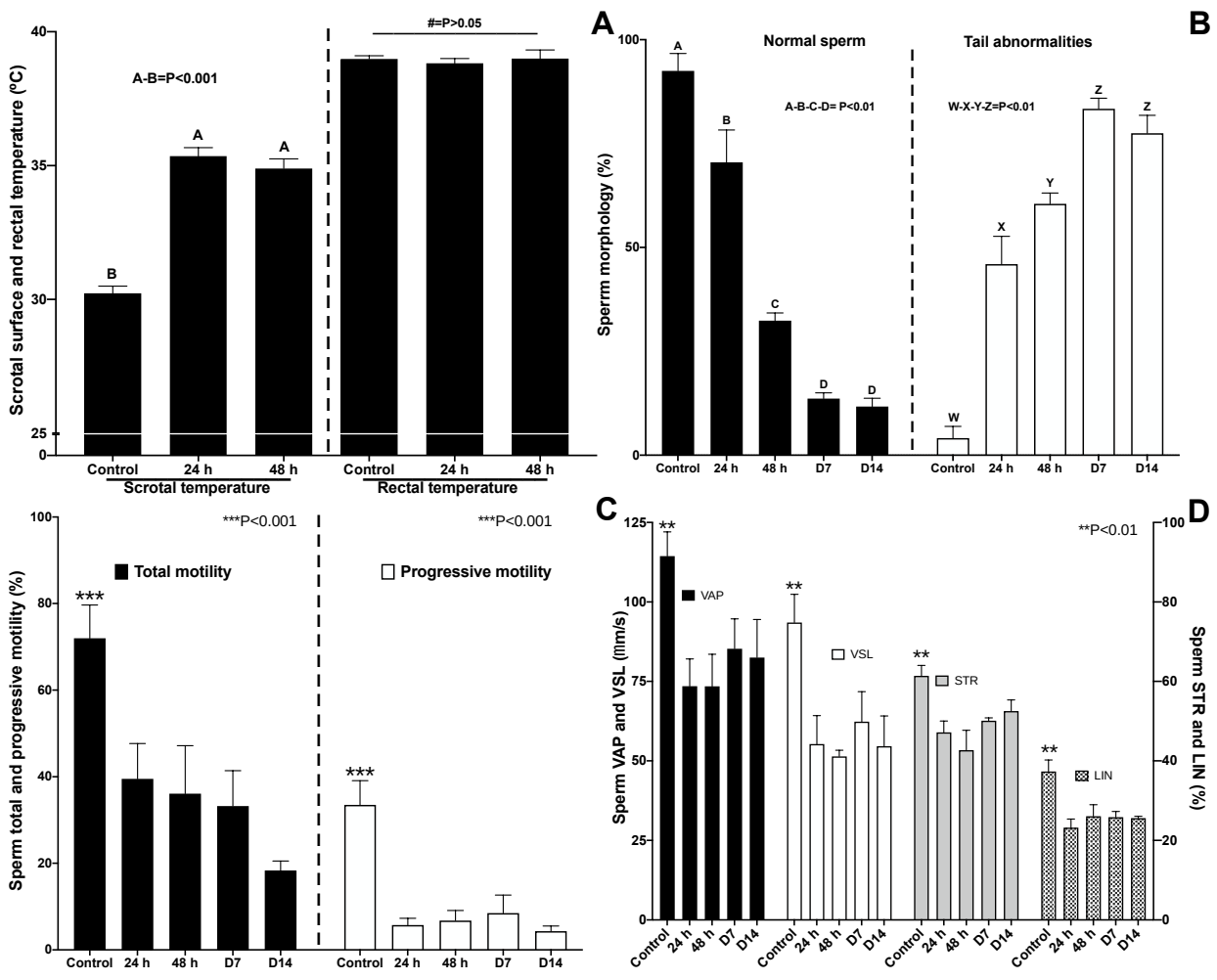

Figure 1. Mean ± SEM scrotal surface temperature and epididymal sperm quality parameters at various intervals after 24 or 48 h of heat stress induced by scrotal insulation in rams (24 h, 48 h, D7, and D14); control rams were not insulated. (A) Scrotal surface and rectal temperatures; (B) morphology of epididymal sperm; (C) total and progressive motilities, and (D) average path (VAP), curvilinear (VCL) sperm velocities, and straightness (STR) and linearity (LIN). Within an end point, means without a common superscript differed (P<0.05; P<0.01 or P<0.001).

For normalized testicular weight (Fig. 2A) and seminiferous tubule diameter (Fig. 2B) there were no differences (P > 0.05) among experimental groups except that the 14D group were notably lower (P < 0.05 and P < 0.001, respectively). The seminiferous tubule histological score (Johnsen, 1970) had a reduction (P<0.05) in quality from 24 h to 7 d, with more severe impairment at 14 d post (P<0.05; Fig. 2 C and Fig. 3) whereas the number of spermatids/g of testes (Fig. 2 D) were lower (P < 0.001) in all insulated groups compared to the Control.

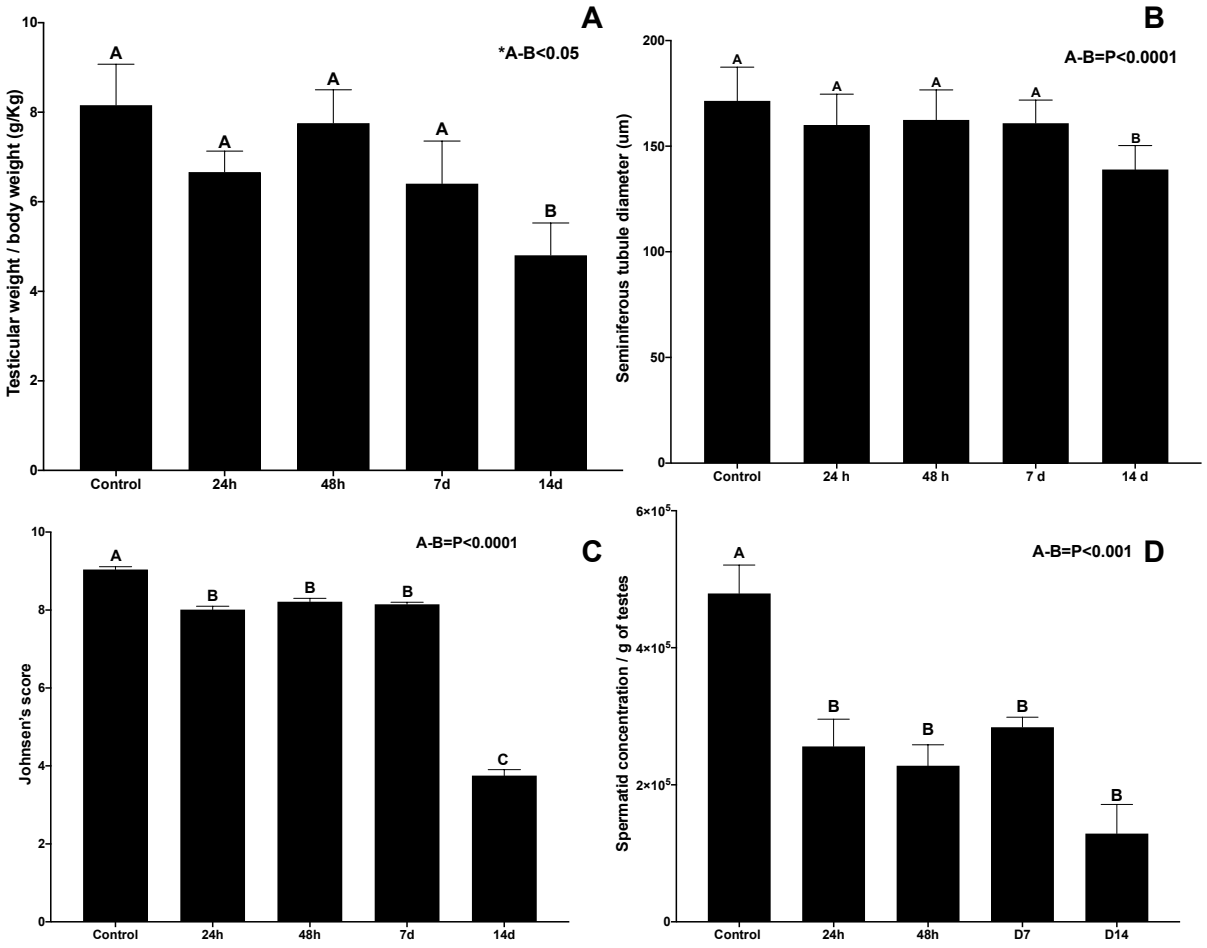

Figure 2. Mean ± SEM for (A) testicular weight/body weight and (B) seminiferous tubule diameter. Within an end point, means without a common superscript differed (P<0.05; P<0.01 or P<0.001); (C) Johnsen's score of seminiferous tubules and (D) Spermatids per gram of testicular parenchyma at various moments after heat stress induced by scrotal insulation in rams (24H, 48H, D7, and D14); control rams were not insulated. Within an endpoint, means without a common superscript differed (P<0.05; P<0.01 or P<0.001).

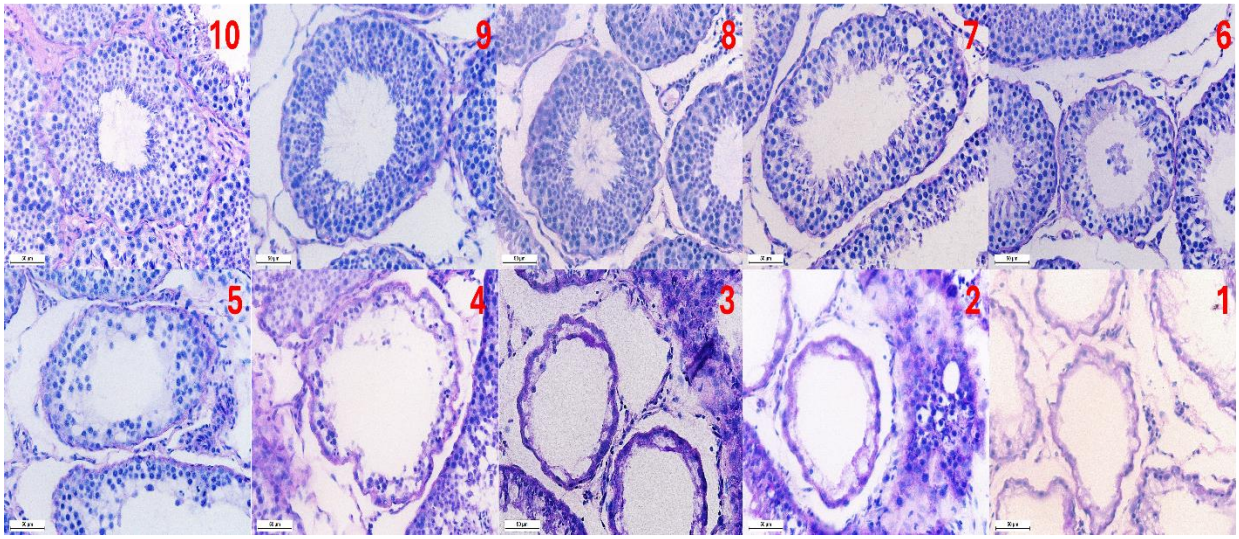

Figure 3. Histological classification of seminiferous tubules based on the Johnsen Score (Johnsen 1970). 10 - Full spermatogenic cycle with plethora of sperm in lumen; 9 – Several sperm in the lumen, with slight disorganization of epithelium; 8 – Few sperm in the lumen; 7 – Absence of sperm but abundance of spermatids; 6 – Absence of sperm and only few spermatids; 5 – Absence of sperm and spermatids with abundance of spermatocytes; 4 – Absence of sperm and spermatids with minimal spermatocytes; 3 – Spermatogonia are the only cells observed; 2 – Sertoli cell only epithelium; 1 – Total absence of cells in tubules.

Discussion

Based on the increased scrotal surface temperature induced by scrotal insulation, the negative temperature gradient between the body and testes (−4°C) was greatly diminished or entirely lost, with testicular and epididymal temperatures approaching body temperature (~39 ºC; Waites, 1962). This temperature increase caused a sharp decline in epididymal sperm quality from 24 h to 7 d post-insulation and also affected the final stages of spermatogenesis (manifested at 14 d post insulation), with significant reductions in spermatid counts. Therefore, our hypotheses were supported; HS induced by scrotal insulation had immediate deleterious impacts on epididymal sperm as well as developing sperm, decreasing sperm production and significantly reducing both STD and testicular weight. Importantly, this is apparently the first report in rams of such an immediate response in sperm kinetics and morphology.

The first effect of epididymal HS induced by scrotal insulation on sperm function was a sharp decrease in total and progressive motility, starting 24 h after the onset of scrotal insulation. Additionally, key characteristics of sperm movement, such as sperm velocity, linearity (LIN), and straightness (STR), also significantly declined. These effects were also evident in groups castrated at 48 h or 7 d post-insulation, indicating that epididymal HS affected all sperm stored in or transiting through the epididymis during scrotal heating, which is a novel finding. Several factors can impair epididymal function impacting sperm motility, including androgen receptor (AR) deficiency (Zhang et al., 2019), reduced miRNA, and disturbances in sperm calcium (Ca2+) and other ion influx/efflux (Brandenburger et al., 2011; Hu et al., 2018; Zhang et al., 2018). Further studies are needed to elucidate the pathophysiology of epididymal HS on sperm motility.

In addition to molecular processes, motility is also dependent on sperm morphology. In groups with lower sperm motility, there were reductions in the percentage of morphologically normal sperm. Although this decrease was already significant in the 24H group, it was progressively more profound in the other experimental groups (48H, 7D, and 14D), with tail defects (mainly bent or coiled tails) being the main sperm defect. In rats, long-term effects of HS depressing sperm motility were associated with downregulation of CatSper-1 and -2 mRNA, and protein expression levels in testicular tissue at 1, 14, and 35 d after HS (El-Eman et al., 2023). Downregulation of these genes may impede sperm motility and hyperactivity (Sun et al., 2017)

Similar to present results, in rats exposed to HS (30 or 43ºC/day for 6 d), epididymal sperm were severely impaired, with lower motility and increased morphological abnormalities as soon as 8 d after the onset of HS (El-Eman et al., 2023). As epididymal transit in rats takes ~9 d to complete (Fernandez et al., 2008), HS in rats also affected epididymal sperm. Additionally, HS in other species indicated results similar to those described here for epididymal sperm,

supporting the assertion that already-formed sperm could be impacted (Garcia-Oliveros et al., 2022; Ahmad et al., 2012).

Epididymal transit induces various changes in sperm membrane composition, permeability, and metabolism (Hammerstedt et al., 1979; Inskeep and Hammerstedt, 1982). Impacts of HS induced by scrotal insulation on epididymal sperm were likely related to disruption of micro-environmental homeostasis. In mice, HS damaged membranes in epididymal sperm immediately after exposure (Wechalekar et al., 2010). Furthermore, acute HS exposure has been linked to alterations in the epididymal proteome, reducing expression of proteins crucial for sperm maturation (Wang et al., 2015). Additionally, HS can impair sperm mitochondrial function and ATP synthesis (Gong et al., 2017) or redistribute sperm subpopulations within the ejaculate (Maya-Soriano et al., 2015). Emerging studies also highlight the role of microRNAs (miRNAs) in the epididymis and indicate that their types and abundance can be influenced by various adverse factors (reviewed by James et al., 2020). Effects of HS on the miRNA profile in the epididymis is a promising area for future studies.

In contrast to our results, in a study with rams subjected to scrotal insulation for 24 h, there were no differences, compared to controls, in any sperm motility end points measured by CASA or in plasma or acrosomal membrane integrity (Hamilton et al., 2016). As deleterious effects of HS depend on intensity of the heat load (Kastelic et al., 1997; Rizzoto et al., 2020C), a possible explanation for this difference is scrotal surface temperature, which was ~ 2 ^{o}C higher in our study, compared to ~ 33 ^{o}C in the previous study.

As previously mentioned, despite the duration of insulation being limited to 48 h, its deleterious effect on epididymal sperm quality was still present 12 d after it ended. At this moment, in addition to epididymal dysfunctions, it also reflects the HS effect on cells on the last stages of spermiogenesis in seminiferous tubules (Rockett et al., 2001). These results were

similar to those reported in ovine ejaculates collected after HS (Kastelic et al., 2017; Alves et al., 2016).

Testicular impairment post-HS exposure is linked to increased oxidative stress, an imbalance in the antioxidant system (Fraczek et al., 2020), and activation of apoptotic pathways leading to testicular cell death (Rizzoto et al., 2020A; B). Importantly, HS induced by scrotal insulation also decreased testicular weight and STD in 14D rams. These changes were preceded by a decrease in the number of spermatids/g of testicular parenchyma and decreased histological integrity of tubules, consistent with a previous statement that spermatids are particularly affected by HS (Rockett et al., 2001). Additionally, the decreased testicular weight implied apoptosis of other germ cells, as they constitute the majority of testicular cells (Wechalekar et al., 2008).

Apoptosis is an elegant physiological process that destroys and removes defective cells and recycles their constituents without promoting tissue inflammation (Elmore et al., 2007). Excessive production of reactive oxygen species and consequent intracellular oxidative stress status and disruption of the protein and DNA integrity, considered a critical consequence of HS on spermatogenic cells, seem to have a primary role in the pathophysiology of HS-induced apoptosis (Rizzoto et al., 2020A; Ohta et al., 2003; Durairajanayagan et al., 2015).

As the interval from apoptosis initiation to completion can be as short as 2–3 h (Elmore, 2007), apoptosis seemed to be a reasonable explanation for the sharp decrease in the number of spermatids in the testicular parenchyma in the 24H rams and the other insulated groups (48H, 7D, and 14D). In rats, increased testicular levels of BAX transcripts, an apoptosis regulator, were associated with immediate and mediated reductions in testis weight after HS (El-Eman et al., 2023).

When considering reductions in testicular weight and STD at 14 d post-exposure (Fig. 2A and B), the time frame for the observation matched the literature, which described major impacts 7 or 14 d post-HS exposure, as at the time of HS, these sperm were at the spermatid stage, the

spermatogenic stage most sensitive to higher temperature exposure (Rockett et al., 2001). Importantly, cells from the spermatogenic lineage constitute most of the testis, and by consequence its weight, which is also strongly related to STD diameter (Wechalekar et al., 2008). A very plausible explanation for the results was activation of p53-intrinsic and extrinsic apoptotic pathway triggered by HS (Rizzoto et al., 2020A; Ohta et al., 2003; Durairajanayagan et al., 2015) prompting apoptosis of spermatids and impaired spermatogenesis.

Potential limitations for the study could be related to: 1) the insulation method, that although very effective in producing HS, does not closely mimic typical conditions during a warm environment, due to fluctuations in ambient temperature and capability of scrotal thermoregulation; and 2) the short evaluation interval (14 d) that represents an important response timeframe but lacks a full spermatogenic cycle for observation. Therefore future studies with animals exposed to increased ambient temperature or a thermal chamber and with more prolonged observations could be interesting alternatives for future studies.

Conclusion

In conclusion, HS induced by scrotal insulation in rams severely impaired epididymal sperm quality, reduced STD, decreased testicular weight, and diminished spermatid populations. The immediate findings at 24 and 48 HS, demonstrated that epididymal sperm were also adversely affected by HS exposure. Furthermore, the observations at 7 and 14 d indicated that spermatogenesis was significantly disrupted, with spermatids being most susceptible.

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Data availability

The raw data supporting the conclusions of this article will be provided by the authors upon request, without reservations.

Author contributions

Teixeira MB: Conceptualization; Investigation; Writing – original draft; **Ferreira JCP**: Conceptualization; Funding acquisition; Supervision; Writing – review and editing. **Codognoto VM**: Investigation; Methodology. **Rossi ES**: Investigation; Methodology. **Pupulim AGR**: Investigation; Methodology. **Carvalho JC**: Investigation; Methodology. **Rates PZ**: Investigation; Methodology. **Oba E:** Investigation; Validation; Methodology; Resources. **Navolar FMN:** Validation; Methodology. **Di Santis GW:** Validation; Methodology. **Kastelic JP**: Conceptualization; Writing – review and editing. **Van Soom A**: Conceptualization; Resources; Writing – review and editing. **Rizzoto G**: Conceptualization; Investigation; Formal analysis; Data curation; Supervision; Writing – review and editing

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