Pathogenesis and mitigation of the deleterious effects of heat stress on bull reproduction

Patogênese e mitigação dos efeitos deletérios do estresse térmico na reprodução de touros

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Abstract

Bull testes must be 2 to 6 ^0C below body temperature for morphologically normal, motile and fertile sperm. Scrotal/testicular thermoregulation is complex, including a coiled testicular artery, surrounded by the venous pampiniform plexus comprising the testicular vascular cone, a counter-current heat exchanger. In addition, heat radiation from the scrotum, sweating, complementary arterial blood supplies, and temperature gradients in the scrotum and testes all contribute to testicular cooling. Despite a long-standing paradigm that mammalian testes are close to hypoxia and blood flow does not increase in response to testicular heating, in recent studies in mice, rams and bulls, warming the testes stimulated increased blood flow, with no indications of testicular hypoxia. Furthermore, hypoxia did not replicate the changes and hyperoxia did not provide protection. Therefore, we concluded that testicular hyperthermia and not secondary hypoxia affects spermatogenesis and sperm quality. Increasing testicular temperature causes many cellular and subcellular changes. As testicular temperature increases, the proportion of defective sperm increases; recovery is dependent upon the nature and duration of the thermal insult. Environmental control of temperature (shade, sprinklers, air conditioning) and some chemical approaches (e.g., melatonin and L-arginine) have promise in reducing the effects of heat stress on bull reproduction.

Key words: Scrotal/testicular thermoregulation; heat stress; scrotum; testes

Resumo

Os testículos dos bovinos devem permanecer 2 a 6 ºC abaixo da temperatura corporal para produzirem espermatozoides morfologicamente normais, móveis e férteis. A termorregulação escrotal/testicular é complexa e envolve a enovelada artéria testicular circundada pelo plexo venoso pampiniforme, que constituem o cone vascular, um sistema contracorrente de troca de calor. Adicionalmente, a perda de calor por radiação pelo escroto, sudorese, suprimento sanguíneo arterial complementar, e os gradientes de temperatura no escroto e testículos contribuem para o resfriamento testicular. A despeito do duradouro paradigma de que os testículos estão em uma situação de quase hipóxia e que o fluxo sanguíneo não aumenta em resposta ao aquecimento testicular, em recentes estudos em camundongos, carneiros e touros, o aquecimento testicular estimulou o fluxo sanguíneo sem serem observados sinais de hipóxia. Além disso, a hipóxia não afetou os testículos e a hiperóxia não conferiu proteção. Portanto, concluímos que é a hipertermia testicular, e não a hipóxia secundária, que afeta a espermatogênese e a qualidade seminal. O aumento da temperatura testicular causa muitas mudanças celulares e subcelulares. À medida que a temperatura aumenta, a proporção de espermatozoides defeituosos aumenta. A recuperação depende da natureza e duração do insulto térmico. O controle ambiental (sombra, aspersores de água e ar condicionado) e algumas abordagens químicas (ex., melatonina e L-arginina) são medidas promissoras de redução dos efeitos do estresse térmico na reprodução de touros

Palavras chave: Termorregulação escrotal/testicular; estresse térmico; escroto; testiculos

Introduction

Bull fertility is relatively more important than cow fertility, as one bull may breed 20 to 30 cows by natural service, or thousands by artificial insemination. Few bulls are sterile, but there is a wide range in fertility (Cates, 1975). Bull testes must be 2 to 6 ^{0}C below body core temperature; increased testicular temperature reduces sperm motility, percentage of morphologically normal sperm, and fertility. There was a long-standing belief that mammalian testes are close to hypoxia, increased testicular temperature does not change blood flow, and hypoxia is responsible for decreases in motility, morphology and fertility. However, recent studies indicated that hyperthermia, not a secondary hypoxia, causes heat-induced changes in sperm.

Anatomy and physiology of scrotal/testicular thermoregulation

Many anatomical and physiological factors keep testes cool. Above the testis, the testicular artery is highly coiled and surrounded by the venous pampiniform plexus, comprising the "testicular vascular cone" (TVC). Blood in the artery flows ventrally towards the testis, whereas blood in the vein flows dorsally, creating a "counter-current" heat exchanger that cools arterial blood. In addition, much heat is lost from the TVC by radiation from the scrotal skin. The TVC and scrotal skin temperatures in *Bos taurus* bulls from 0.5-3 y have been described (Cook et al., 1994). The skin covering a bull's scrotum is usually thin, hairless and with many blood vessels that dilate or constrict to regulate blood flow and temperature (Setchell, 1978). A long scrotal neck allows the testes to move ventrally, although an extremely long scrotal neck may make testes more susceptible to injury. Location of the testes is regulated by both the tunica dartos and the cremaster muscles, which are smooth and striated muscles, respectively.

Sweating and whole-body responses contribute to testicular cooling and have been best characterized in sheep. In Merino rams, scrotal sweat glands are larger and produce more sweat than those elsewhere on the body (Waites and Voglmayr, 1962). Similarly, sweat gland density is higher in scrotal skin than any other body region in bulls (Blazquez et al., 1988). Apocrine sweat glands in the scrotum of rams discharge simultaneously; expulsion begins when scrotal surface temperature is ~ 35.5 ^{0}C, with up to 10 discharges per hour (Waites and Voglmayr, 1963). Whole-body responses in rams include rapid breathing when scrotal surface temperature rises above 35 - 36 ^{0}C (Setchell, 1978). Furthermore, when scrotal surface temperature in rams reaches 38 - 40 ^{0}C, respiration becomes very rapid (e.g., 200 breaths per minute), there is peripheral vasodilation and rectal temperatures can decline 2 ^{0}C in 1 h (Waites, 1962)

Surface and internal temperatures

In 16 crossbred *B. taurus* beef bulls (Kastelic et al., 1995), temperatures were measured at three locations: top, middle and bottom of the testis. Average temperatures (^{0}C) at these locations were: 30.4, 29.8 and 28.8 (scrotal surface); 33.3, 33.0 and 32.9 (scrotal subcutaneous); and 34.3, 34.3 and 34.5 (intratesticular). Top-to-bottom temperature differences (gradients) were 1.6, 0.4 and -0.2 ^{0}C for scrotal surface, scrotal subcutaneous and intratesticular temperatures, respectively. Therefore, the temperature gradient was most pronounced on the scrotal surface, small in scrotal subcutaneous tissues, and essentially absent in the testicular parenchyma.

In bulls, caput, corpus and cauda epididymis temperatures averaged 35.6, 34.6 and 33.1 ^{0}C respectively, and the gradient between the caput and the cauda averaged 2.5 ^{0}C (Kastelic et al., 1995). The temperature of the caput was greater than that of the testicular parenchyma at the top of the testis, probably because the caput is close to the TVC. However, the cauda, an important site for sperm storage and maturation, was slightly cooler than the testicular parenchyma.

Factors underlying scrotal/testicular temperatures

To determine the relative importance of blood flow versus metabolism as sources of testicular heat, blood flow to the testes and intravascular temperatures and blood gases in the testicular artery and vein were determined in eight Angus bulls (Barros et al., 2018). Average flow in the testicular artery was 12.4 mL/min. Arterial blood was warmer (39.2 versus 36.9 ^{0}C, P<0.001) with more saturated hemoglobin than blood in the testicular vein (95.3 versus 42.0%, P<0.001). Based on blood flow and hemoglobin saturation, each testis used 1.2 mL per minute of O_2 to produce an average of 5.8 calories of heat. In contrast, based on blood flow and the arterial-venous temperature difference, blood flow contributed 28.3

calories per minute. Therefore, blood flow was the major source of testicular heat.

To determine contributions of the scrotum and testes to scrotal/testicular thermoregulation, a novel model was created, with bilateral scrotal incisions, leaving the testicular cords intact and holding one testis outside the scrotum (Kastelic et al., 1996a). Temperature gradients (top minus bottom temperature, ^{0}C) were 2.1 and 2.5 on the scrotal surface, with and without a testis, respectively, and within the testis, gradients were -0.2 with the scrotum and -0.6 for the bare testis (no scrotum). Therefore, the scrotum had a positive temperature gradient (warmer top), but for the testis, the gradient was negative (warmer bottom), with the two gradients being complementary and testicular temperature being below body core temperature.

In another study (Kastelic et al., 1997), we explored the role of blood vessels in scrotal/testicular thermoregulation. After the blood in the testicular artery is cooled in the TVC cone, the testicular artery goes to the bottom of the testes, branches, and then goes dorsally before entering the testicular parenchyma. There was no significant difference in blood temperature between the bottom of the TVC and the bottom of the testis, but blood cooled before entering the testes. To the best of our knowledge, the arterial supply to the scrotum is from dorsal to ventral. Therefore, for both the scrotum and testes, arterial blood is warmest at the origin of the supply (top of scrotum, bottom of testis) and cooler furthest from the origin of the supply (bottom of scrotum, top of testis), and scrotal and testicular arterial blood supplies had complementary gradients. Development of the TVC and its association with scrotal thermoregulation, semen quality and sperm production were reviewed (Kastelic *et al.,* 2018).

Comparisons between *B. indicus* and *B. taurus* bulls

Testicular thermoregulation and various aspects of the scrotum and TVC were studied in *B. indicus*, *B. taurus* and crossbred bulls (Brito et al., 2004). There were profound differences, particularly between *B. indicus* versus *B. taurus* bulls, in several aspects of scrotal/testicular thermoregulation. It appeared that TVC characteristics may make *B. indicus* bulls less susceptible to increased ambient temperatures by having a better blood supply for the testes and more efficient heat transfer between the testicular artery and vein. In a study of 107 *B. indicus*, B. *taurus* and crossbred bulls (Brito *et al.,* 2002), age and genetics affected the scrotum, testes, and TVC, sperm production and quality and that some characteristics of the scrotum, testes and TVC had associations with sperm production and quality.

Evaluation of scrotal surface temperature with infrared thermography

Infrared thermography has been used as a rapid and non-invasive method to assess scrotal/testicular thermoregulation. Scrotal thermograms of "normal" bulls had the following characteristics: warmest over the TVC, decreasing from below the TVC to the bottom of the scrotum, and similar temperatures horizontally across the scrotum (Purohit et al., 1985). Other patterns that were less consistent, for example, not similar when comparing left to right, and areas of uniform, high temperature (not over the TVC) were considered abnormal thermoregulation of the testis or epididymis. Most bulls with an abnormal scrotal thermogram had reduced semen quality (Purohit et al., 1985); however, some bulls with poor semen had a normal thermogram. In bulls with unilateral orchitis, scrotal surface temperature was higher over the affected testis. In rams, scrotal surface temperature was highly correlated with temperature in the scrotal subcutaneous tissues and with a "model testis" (water-filled balloon; Coulter et al., 1988). Although infrared thermography is a rapid and non-invasive assessment, scrotal surface temperature may not exactly represent temperature of the underlying testis (Kastelic *et al.,* 1995). Environmental factors affecting the use of infrared thermography for assessment of scrotal surface temperature have been reported (Kastelic et al., 1996b)

To assess scrotal surface temperature in breeding soundness evaluation (Lunstra and Coulter, 1997), 30 beef bulls (~1 y of age), all judged satisfactory on a standard breeding soundness examination, were used for breeding (each bull with ~ 18 heifers for 45 d). For bulls with a scrotal thermogram regarded as normal or doubtful, pregnancy rates (80 d after the end of the breeding season) were 83 and 85%, respectively, higher (P<0.01) than those for bulls with an abnormal scrotal thermogram (68%).

Increased ambient temperature

There are many reports documenting how increased ambient temperature decreases sperm quality. In two Guernsey bulls, 37 ^{0}C and 81% relative humidity 12 h per day for 17 d reduced the

number of sperm, sperm concentration, motility and normal sperm, with ~ 30-40% abnormal sperm) (Casady et al.,1953). Furthermore, it is well known that *B. indicus* bulls are much less susceptible than *B. taurus* bulls to decreased sperm quality due to increased ambient temperature (Skinner and Louw, 1966).

Scrotal insulation

Scrotal insulation is a common model to study the effects of increased testicular temperature. Insulating the scrotum (48 h; insulation = Day 0) of *B. taurus* x *B. indicus* bulls caused the following abnormalities; decapitated sperm on Days 6 to 14; sperm with abnormal acrosomes on Days 12 to 23; abnormalities of sperm tails on Days 12 to 23; and sperm with droplets on Days 17 to 23 (Wildeus and Entwistle, 1983) Based on the interval from scrotal insulation to appearance of morphological abnormalities, there were effects on spermatids, as well as sperm in the caput epididymis. Despite no effect on daily sperm production, there was nearly a 50% reduction in the number of sperm in the epididymis, likely due to resorption of morphologically abnormal sperm.

In six Holstein bulls with 48 h of scrotal insulation (start of insulation = Day 0), there was no change in number of sperm collected, but progressively motile sperm, 69% before insulation, was only 42% on Day 15 (Vogler et al., 1991). There were 80% morphologically normal sperm from pre-insulation to Day 9, but then decreased to 53% on Day 12 and 14% on Day 18. Despite variations among bulls in the percentage of abnormal sperm, the timing of appearance of specific abnormalities was quite consistent, similar to another study that used either scrotal insulation or dexamethasone treatment to interfere with spermatogenesis (Barth and Bowman, 1984). Furthermore, when sperm were frozen and thawed and then incubated for 3 h at 37 ^{0}C, sperm collected prior to insulation had higher progressively motility (46 vs 31%, respectively) and intact acrosomes (73 vs 63%) compared to semen collected 3, 6, or 9 d after the start of scrotal insulation (Vogler et al., 1993). Therefore, although sperm appeared morphologically normal, cryopreservation and thawing revealed changes in sperm in the epididymis during insulation.

Although whole-scrotum insulation has been widely used, there are also reports of insulating only the scrotal neck. In one study (Kastelic et al., 1996c), scrotal neck insulation was done for 7 d (Days 0 to 7) as a model for excessively fat bulls (usually with much fat in the scrotal neck). Sperm in the epididymis or in acrosomal phase were apparently most affected, with increased abnormalities starting soon after insulation and peaking around 20 d after the start of insulation, then nearly returning to pre-insulation values by 35 d after the start of insulation. In another study (Brito et al., 2003), scrotal neck insulation in *B. indicus* x *B. taurus* bulls did not significantly affect semen quality. In a recent study in *B. taurus* bulls and rams (Shahat et al., 2021), scrotal subcutaneous temperature was significantly increased by insulating the scrotum or increasing ambient temperature, whereas it was not significantly affected by scrotal neck insulation; however, all three heat-stress models decreased sperm motility and morphology.

Hypoxia versus hyperthermia

Despite a long-standing paradigm that mammalian testes are close to hypoxia and blood flow does not increase in response to testicular heating, limited data support this idea. We conducted a 2 x 3 factorial study in rams with testicular insulation (yes/no) and three O_2 concentrations (14, 21 and 85%) in inspired air for 30 h (Kastelic et al., 2017). Semen was collected twice weekly for 4 wk, then once weekly for 2 wk. There were primarily deleterious effects of increased testicular temperature on sperm motility and morphology, and failures of hyperoxia to prevent these changes or hypoxia to replicate them. Therefore, we concluded hyperthermia and not hypoxia caused deleterious effects of increased testicular temperature on spermatogenesis and sperm quality. In another study of the hypoxia paradigm (Kastelic et al., 2019), we used 48 mice in a 2 x 3 factorial study, exposing them to ambient temperatures of 20 or 36 °C and environments with 13, 21, or 95% O_2 on two occasions for 12 h, (separated by 12 h at 20 °C and 21% O_2), with sperm collected and evaluated 14 or 20 d after first exposure. There were mainly effects of hyperthermia; mice exposed to 36 ^{0}C had significantly lighter testes (96.9 vs 110.2 mg), lower daily sperm production (21.1 vs 24.7 × 10^6 sperm/g testes), and fewer motile (41.5 vs 54.5%) and morphologically normal sperm (45.4 vs 59.9%), plus significant differences in testicular histology. In addition to hyperthermia being responsible for nearly all deleterious effects, as in the rams, these effects were not replicated by hypoxia nor prevented by hyperoxia.

To further explore the role of hypoxia versus hyperthermia, we conducted three additional studies. In the first study, in eight anesthetized rams, O_2 concentration in inspired air was decreased from

100 to 21 and eventually 13% (Rizzoto et al., 2018). In these rams, testicular blood flow increased (9.6 ± 1.7 vs 12.9 ± 1.9 ml/min/100 g of testis, P<0.05). Increased testicular blood flow maintained O_2 delivery and increased testicular temperature by ~1 ^{0}C, but there were no indications of testicular hypoxia. In the second study, testes of nine anesthetized rams were increased from 33 to 37 to 40 ºC (Rizzoto et al., 2019). As testicular temperature increased from 33 to 40 ºC, there were increases in testicular blood flow (13.2 ± 2.7 vs 17.7 ± 3.2 ml/min/100 g of testes, P<0.05), O_2 extraction (31.2 ± 5.0 vs 47.3 ± 3.1%; P<0.0001) and O_2 consumption (0.35 ± 0.04 vs 0.64 ± 0.06 mL/min/100 g of testes; P<0.0001). In the third experiment, *B. indicus* and *B. taurus* bulls were maintained under general anesthesia, and testes were warmed from 34 to 40 °C (Rizzoto et al., 2020a). Testicular warming caused significant increases in testicular blood flow (9.59 ± 0.10 vs 17.67 ± 0.29 mL/min/100 g, respectively), delivery of O_2 (1.79 ± 0.06 vs 3.44 ± 0.11 mL O_2/min/100 g) and consumption of O_2 (0.69 ± 0.07 vs 1.25 ± 0.54 mL O_2/min/100 g), with no significant differences between breeds. In all three experiments, there was no evidence of anaerobic metabolism, based on a lack of change in lactate, pH, HCO_3-, and base excess. In conclusion, these data challenged the paradigm regarding scrotal/testicular thermoregulation, as acute testicular hyperthermia increased blood flow and increased O_2 delivery and uptake, with no indication of hypoxia or anaerobic metabolism. These and other studies have been reviewed (Rizzoto and Kastelic, 2020b).

Effects of increased temperature on testicular cells

Increased testicular temperature affects all testicular cells, including Sertoli and Leydig cells, although germ cells are most affected (Waites and Setchell, 1990). All spermatogenesis stages are susceptible, with degree of damage dependent on the extent and interval of increased testicular temperature (Waites and Setchell, 1990). Spermatocytes in meiotic prophase are very sensitive, whereas more mature sperm usually have changes in metabolism and/or morphology (Setchell et al., 1971). Despite variations among individual bulls in the kind and percentage of abnormal sperm, defects appear in a predictable order (Wildeus and Entwistle, 1983; Barth and Bowman, 1994). Sperm morphology usually returns to normal within ~ 6 wk after increased testicular temperature, although a severe and/or prolonged increase in testicular temperature will delay recovery.

There are some studies to understand the effects of testicular hyperthermia at the cellular and molecular levels. For example, short-term scrotal insulation in *B. indicus* bulls caused: profound reductions in testicular testosterone concentrations; increased expression of antioxidant molecules (GPX1) and upregulation of chaperones (HSP70); and down-regulation of StAR and BCL-2, fundamental for spermatogenesis and sperm quality and anti-apoptotic activity, respectively (Rizzoto et al., 2020c). In a subsequent study with acute testicular warming in mice (Rizzoto et al., 2020d), there was: involvement of the chaperone and antioxidant systems (upregulation of HSP70 and GPX1, respectively); and upregulation of P53 and modulation of factors associated with the P53-dependent apoptotic pathway (intrinsic and extrinsic routes, BCL2 and CASP 8, respectively). A summary of the suggested pathways of heat stress impacts and responses in testes is included in a recent review (Shahat et al., 2020).

Mitigation of the effects of testicular hyperthermia

In brief, there are several methods used to mitigate the effects of testicular hyperthermia. The first approach is to minimize the extent and duration of thermal stress (see Ferreira et al., 2021), by providing shade, sprinklers or perhaps air-conditioned stalls for valuable bulls in AI centres. It is well known that *B. indicus* bulls will generally be much less susceptible to the effects of increased ambient temperatures on reductions in sperm quality than *B. taurus* bulls; therefore, choosing breeds and biological types best suited to the conditions is important. For reasons that are not entirely clear, among a group of bulls, even within a breed, there is a substantial range in the extent to which they are affected by increased ambient temperature, providing some opportunity for genetic selection. Finally, there have been many attempts to either add compounds (e.g., antioxidants) to semen extender, or to give these compounds to bulls (reviewed in Shahat et al., 2020). Some of these have had some promise, with very encouraging results emerging, especially with melatonin. Recent advances in understanding how increased testicular temperature affects spermatogenesis and sperm quality, both at the whole-animal and cellular/molecular levels, should facilitate evidence-based approaches to reducing or minimizing these effects.

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