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Heat stress responses in spermatozoa: Mechanisms and consequences for cattle fertility

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ABSTRACT

Currently, the world is facing the negative impact of global warming on all living beings. Adverse effects of global warming are also becoming obvious in dairy cattle breeding. In dairy bulls, low fertility has frequently been reported during summer season especially in tropical or subtropical conditions. Typically, spermatozoa at post-meiotic stages of development are more susceptible to heat stress. During this period extensive incorporation of histone modifications and hyperacetylation turns the chromatin into an unstable conformation. These unstable forms of chromatin are thought to be more vulnerable to heat stress, which may have an effect on chromatin condensation of spermatozoa. Spermatozoa with altered chromatin condensation perturb the dynamics of DNA methylation reprogramming in the paternal pronucleus resulting in disordered active DNA demethylation followed by *de novo* methylation patterns. In addition, there was a tendency of decreased size in both paternal and maternal pronuclei after fertilization of oocytes with heat-stressed spermatozoa, leading to lower fertilization rates. In this review, we will focus on the mechanisms of heat stress-induced sperm defects and provide more detailed insights into sperm-borne epigenetic regulations.

1. Introduction

Global warming and its effects on climate change is unanimously accepted reality for the world. Climate change has been noticeable throughout the globe over the past several decades, irrespective of geographical boundaries. These changes are not only limited in the tropical or subtropical regions but are also visible in the temperate regions [1]. South Asian regions rely mainly on agriculture and small-scale livestock farming and are considered to be extremely vulnerable to climate change. High temperature on one hand eventually reduces yields of agricultural crops and on the other hand encourages weed and pest proliferation. Therefore, multidimensional effects have been projected in the Intergovernmental Panel on Climate Change (IPCC) [1] including increase in frequency of hot extremes, heat waves, tropical cyclone intensity and extra-tropical storm tracks, heavy precipitation, etc. Meanwhile, climate change is evident by the increased incidence of extremely hot summers, droughts, tropical cyclones, and flooding in the South Asian regions [2]. In an earlier report [3], it was mentioned that these unprecedented outcomes of climatic change are also noticeable to some extent in Central and Northern Europe and North America. The adverse effects of climate change on animal production are taking place through a number of ways: i) directly affecting animal health and reproduction, ii) indirectly affecting feed and grains production, and iii) increasing exposure to pests and pathogens [4]. The impacts of climate change, especially high ambient temperature on productivity of livestock and dairy animals have been reviewed by Silanikove and Koluman [5]. The authors reported that under conditions of high temperature, animals will lose their appetite, slow down their growth rate, decrease milk and meat production, exhibit a reduced reproductive performance, and show increased sensitivity to diseases and pests [5]. Consequently, the availability of food for human consumption either from animal or plant sources will reduce considerably.

High ambient temperature is considered to be one of the most important factors for subfertility in cattle in tropical or subtropical countries [6,7]. Reduction of fertility in dairy cattle due to heat stress is well documented even in a temperate climate [8]. Heat

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stress has been shown to have a negative effect on both oocytes and early embryos, which compromises with fertilization and/or embryo development [9]. In a similar study, Ealy et al. [10] showed that the early bovine embryos on Day 1 are highly sensitive to heat stress but become resistant after Day 3 of development, which may be due to the production of heat shock proteins (HSPs). Likewise, in bulls, summer season has frequently been reported to deteriorate semen quality even in a temperate climate [11,12]. Summer heat stress is a major concern for semen quality in beef bulls especially Belgian Blue bulls due to their extreme muscularity combined with small scrota and hence a small scrotal skin surface area for heat transduction [13].

Bovine spermatogenesis is a complex and delicate process and is accomplished generally through 3 phases consisting of approximately 61 days in total, from spermatogonia until elongated spermatids [14]. However, it is difficult to establish at which stages of spermatogenesis spermatozoa are most susceptible to heat stress, since the start of exposure of the spermatozoa to heat stress, the duration and the withdrawal from heat stress is difficult to predict. Therefore, in our previous *in vivo* study model, we artificially increased scrotal temperature of two breeds of bulls by scrotal insulation method for a fixed period of 48 h [15,16] and we investigated sperm abnormalities throughout the spermatogenic cycle. In our study, we observed that germ cells at post-meiotic stages of development are more susceptible to heat stress and the resulting spermatozoa are associated with altered chromatin conformation possibly due to aberrant DNA-protamination [17]. In a review study, Saacke [18] postulated that sperm chromatin aberrations in morphologically normal or nearly normal ejaculates may lead to uncompensable effect in field fertility. The notion of uncompensable effect refers to the defects of spermatozoa where the conception rates cannot be improved by increasing the number of viable spermatozoa in the insemination dose [19]. As a matter of fact, uncompensable defects of spermatozoa might be related to epigenetic modifications since environmental agents can modify the gene expression profiles without changing their DNA sequence or copy number and these modifications can be transmitted to the offspring [20]. This is well addressed in a recent study where high and low bull fertility were found to be associated with different sperm epigenetic patterns (DNA methylation) that resulted in transcriptional differences in the preimplantation embryos [21].

Preimplantation embryo development is a dynamic process and is regulated by both genetic and epigenetic mechanisms. Epigenetic regulation in the embryos is considered to be caused by DNA methylation, histone modifications, and/or post-transcriptional modifications through microRNA populations (reviewed in Ref. [22]). In cattle, following fertilization, DNA in the paternal pronucleus is actively demethylated followed by *de novo* methylation within the narrow developmental window of the zygotic stage, whereas DNA methylation in the maternal pronucleus remains almost unchanged [23–25]. The typical characteristics of DNA methylation patterns are also evident in many other species and are considered utmost important for early embryonic development [26,27].

Until today, little is known about sperm-derived epigenetic regulations in the resulting embryo. In this review, we will focus on what is already known about bovine testicular development, scrotal shape, scrotal circumference, scrotal and testicular thermoregulation, and bovine spermatogenesis in relation to their vulnerability under heat stress conditions. Next, we will review the mechanisms and concurrent effects of high ambient temperature or induced testicular temperature on semen quality. Finally, we will focus especially on heat-stressed sperm-mediated epigenetic regulations in the developing embryos and the possible mitigation strategies for reducing the effects of heat stress in animal reproduction.

2. Testicular development, scrotal shape and scrotal circumference in relation to better thermoregulation under heat stress environment

2.1. Testicular development and scrotal shape

Postnatal developmental assembly of the testicular tissue and seminiferous epithelium occurs over a period of months and developmental rate varies between cattle breeds. For example, in *Bos taurus* bull calves, testis weight increases from 9 g at 4 weeks to 180 g at 32 weeks of age [28]. On the other hand, in *Bos indicus* bull calves, testis weighs only 20 g at 36 weeks of age [29], which indicates slow testicular growth and delay of puberty in this type of cattle. The scrotal shape has a major influence on further testicular development and function. For a normal testicular function, the testes should be freely movable inside the scrotum. A possible cause of low fertility in bulls is abnormal testicular and/or scrotal sac development [26,30]. Bulls having a normal scrotum with a distinct scrotal neck generally have the best field fertility reports [31]. On the other hand, beef bulls such as Belgian Blue typically have a small scrotum without a distinct scrotal neck [13]. The anatomical peculiarity of small scrotum without a distinct scrotal neck possibly increases the susceptibility of such beef bulls to heat stress leading to low field fertility [32,33]. Scrotal length is categorized in terms of the relative position between the bottom of scrotal sac and the hock joint. Short scrotum is classified as virtually neckless and positioned close to the abdominal wall. Normal scrotum has a distinct scrotal neck and bottom and is positioned above or at the level of hock joint whereas the bottom is pendulated below the hock joint in case of long scrotum. Irrespective of breeds, normal scrotal shape has been reported to be associated with high semen quality [34].

2.2. Scrotal circumference

The most important factor for inadequate semen quality is highly related to incomplete testicular maturation and/or small scrotal shape, which is better predicted by measuring scrotal circumference (SC). Scrotal circumference is measured by tightly fastening a scrotal tape at the middle position of the scrotum after firmly pulling down of the testes to the extremity of scrotal sac. Studies reported that SC may be a more reliable predictor for the onset of puberty than either age or body weight, irrespective of breed [35]. However, Barth and Ominski [36] showed that SC in weaned bulls may not be useful as a culling tool, since a large number of bulls, regardless of breed were below the selected cut-off measurements ($\geq$25 cm). The authors further reported that SC at 240 days may be a useful predictor for selecting bulls with a higher probability of achieving minimum SC at the age of one year [36]. In subsequent studies, SC has been shown as a good indicator of testicular volume and has also been found to have a strong association with the semen output and quality [13]. The minimum SC requirements for *Bos taurus* bull in order to pass breeding soundness evaluation is around 31 cm at the age of 15–18 months [37]. However, *Bos indicus* bulls pass the required SC 31 cm only at the age of 36–48 months [38]. In 1993, The Society for Theriogenology (SFT) published revised standards for selection of breeding bulls termed as Breeding Soundness Evaluation (BSE). According to the SFT, the threshold scrotal circumference (SC) for BSE is the same (34 cm) for bulls of any *Bos taurus* breed of more than 2 years of age. Bulls that do not meet the threshold SC classified as unsatisfactory potential breeders (reviewed in Ref. [39]). In this regard, Hoflack et al. [40] reported that beef bulls (Belgian Blue) younger than 48 months had a below (44%) threshold SC compared to Holstein bulls (17.6%) and showed inferior semen quality, which might be related

to less testicular volume as well as poor scrotal/testicular thermoregulation.

2.3. Scrotal and testicular thermoregulation

Generally in mammals, for normal spermatogenesis the testes require a temperature of 4 °C–5 °C lower than body core temperature [33,41]. This is achieved in most mammals by localization of the testes in the scrotum, outside the abdominal cavity. In bulls, the scrotal skin is thin, devoid of subcutaneous fat and is fairly hairless. The extensive vasculature and lymphatic arrangement of testes with superficial blood vessels of the scrotum may facilitate in removing heat from the testes. Smooth muscles in the cutaneous arterioles of the scrotum are innervated by sympathetic neurons. Stimulation of these neurons by cold causes vasoconstriction; and on the other hand, heat causes a vasodilation of these arterioles and is thereby decreasing or increasing blood supply to the scrotum [42]. Just beneath the scrotal skin, there are two important muscles: the tunica dartos and the cremaster that play a pivotal role in thermoregulation. The tunica dartos is a thin sheet of smooth muscle, which is under tonic control from nerves in the lumbar sympathetic system that promptly positions the scrotum towards the abdomen or away from the abdomen in response to cold and warm environments, respectively [42]. Due to the tonic characteristic of this muscle, the contractile nature can be sustained for a prolonged period especially in cold environment. The function of the cremaster muscle is also to bring the testes close to the abdomen upon contraction. However, due to the striated nature of this muscle, it cannot sustain contraction for a prolonged period of time. Beside these muscular contractions, a major player in cooling the testes is the vascular system. The testicular artery brings warm blood from the body core to the testes and in all farm animals it is tortuous in nature. This tortuous form of artery is intimately entangled by a complex venous network, called the pampiniform plexus and the entire structure (the venous network and artery) is termed the testicular vascular cone [43]. Due to this characteristic vasculature, a countercurrent blood circulation system is evident in the testes (Fig. 1). As a result, the arterial blood entering the testes is cooled to some extent by the venous blood leaving the testes. Moreover, the sweat glands also have an important role in controlling testicular temperature since the density of sweat glands is higher in the scrotal skin than the other body regions in bulls [44]. Beside the protection of the testes, the scrotal skin has a vital role in testicular thermoregulation. Therefore, breeding bulls having normal scrotum with adequate scrotal circumference can cope up with heat stress to some extent.

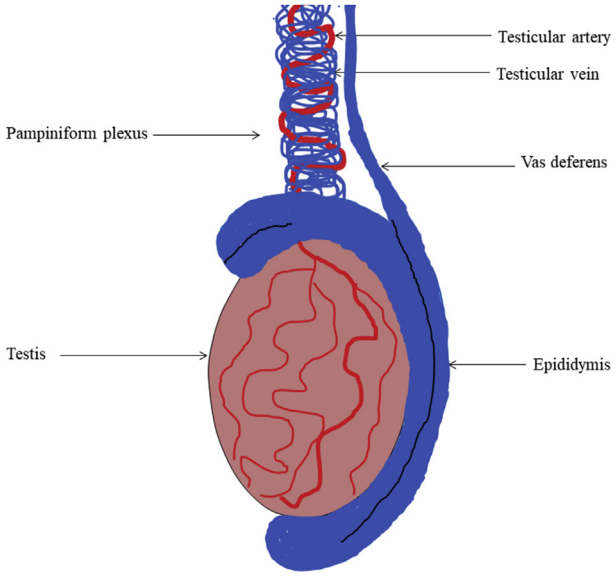

Fig. 1. Model of counter-current heat transfer between the venous blood in the form of pampiniform plexus and the blood of the testicular artery.

2.4. Testicular surface and internal temperature gradient

From the above it is clear that normal testicular function in mammals is related to its specialized cooling mechanisms. Maintenance of the testicular temperature around 32 °C is highly important for normal spermatogenesis. In an elaborate study, Kastelic et al. [45] measured the testicular temperature in 16 crossbred beef bulls at three different positions of the testicle: top, middle, and bottom. The average temperatures at these three locations were recorded as 30.4 °C, 29.8 °C and 28.8 °C (scrotal surface temperature); 33.3 °C, 33.0 °C and 32.9 °C (scrotal subcutaneous temperature); and 34.3 °C, 34.3 °C and 34.5 °C (intratesticular temperature), respectively. From this study it could be concluded that the top-to-bottom temperature differences were 1.6 °C, 0.4 °C and −0.2 °C for the scrotal surface, scrotal subcutaneous, and intratesticular temperatures, respectively. Hence, the temperature gradient was most pronounced on the scrotal surface, trivial in the scrotal subcutaneous tissues, and absent in the testicular parenchyma. In a subsequent study by the same research group, it was reported that the scrotal surface and testes have opposite, complementary temperature gradients, resulting in a relatively uniform intratesticular temperature [31]. This study indicates that the scrotum has a significant influence on maintaining the testicular temperature but the testes have only little influence on scrotal temperature. This is because both scrotum and testes have a characteristic vasculature. The scrotum is vascularized from top to bottom whereas the testicular vascularization is opposite; it is vascularized from the bottom to the top. The testicular artery (after leaving the ventral aspect of the testicular vascular cone) progresses over the length of the testis (under the corpus epididymis), reaches the bottom of the testis, and then diverges into multiple branches that spread dorsally and laterally across the surface of the testis before entering the testicular parenchyma [46]. In a subsequent study, Kastelic et al. [47] showed that there was no difference in temperature of testicular arterial blood at the top of the testis (below the vascular cone) compared to the bottom of the testis, but it was significantly cooler at the point of entry into the testicular parenchyma. The temperature of caput epididymis is higher than that of the testicular parenchyma at the top of the testis, probably because the caput is so close to the testicular vascular cone. However, the cauda epididymis, an important site for sperm storage, is slightly cooler than the testicular parenchyma. Therefore, from the above discussion it is well established that several preset features contribute to the regulation of the testicular temperature. If there is any interruption in these preset mechanisms harsh consequences might be ensued on spermatogenesis.

3. Bovine spermatogenesis

Spermatogenesis denotes the process of development of spermatozoa from primordial germ cells (PGCs). It is a continuous process of PGCs differentiation and development and, in fact, PGCs are derived from a small subset of embryonic epiblast cells. In mammals, after reaching puberty, spermatogenesis is initiated mainly through the influence of bone morphometric protein 8B (BMP8B) [48]. In bulls, spermatogenesis is accomplished through three distinct phases consisting of (i) spermatocytogenesis, (ii) meiosis, and (iii) spermiogenesis that take approximately 21, 23

and 17 days, respectively [14] (Fig. 2).

During spermatocytogenesis, PGCs divide by mitosis to form type A1 spermatogonia. They are the stem cells. An important part of this phase is stem cell renewal. Some type A1 spermatogonia revert back to stem cells and the process continues from which new spermatogonia can be derived [49]. The other type A1 spermatogonia divide progressively by mitosis to form type A2, type A3 and type A4 spermatogonia [50]. The type A4 spermatogonia again divide to form intermediate spermatogonia (type Int) and then to form type B spermatogonia. All of these divisions take place in the basal part of seminiferous tubules. The type B spermatogonia again divide by mitosis at least once or twice to form primary spermatocytes.

Meiosis is completed by two steps: meiosis I and meiosis II. During meiosis I, the primary spermatocytes duplicate their DNA and undergo progressive nuclear changes of meiotic prophase known as preleptotene, leptotene, zygotene, pachytene and diplotene before dividing to form secondary spermatocytes [51]. The secondary spermatocytes undergo a second meiotic division to form haploid cells which are referred to as round spermatids. The meiotic stages of cell division take place in the adluminal compartment (inner side) of the seminiferous tubules [52]. The further process during which the spermatid develops gradually into spermatozoon is called spermiogenesis.

During spermiogenesis, the changes which take place are the condensation of the nuclear chromatin, formation of the flagellum or sperm tail and development of the acrosomal cap [53–55]. This phase also takes place in the adluminal compartment of seminiferous tubules. The resulting elongated spermatids further move close to the lumen of the seminiferous tubules. Lastly, the elongated spermatids are released into the lumen of the seminiferous tubules while they undergo a further transformation process known as spermiation before moving to the epididymis for final maturation and storage [56]. Spermatocytes and/or spermatids undergo extensive remodeling during the meiotic and spermiogenic phases of development: initially nucleoprotein histones are replaced by non-histone transition proteins and finally by protamines [57]. Protamines are involved in packaging of sperm chromatin in such a way that the paternal genome remains functionally inert and protected, and a remarkable reduction in nuclear volume is achieved [58–60].

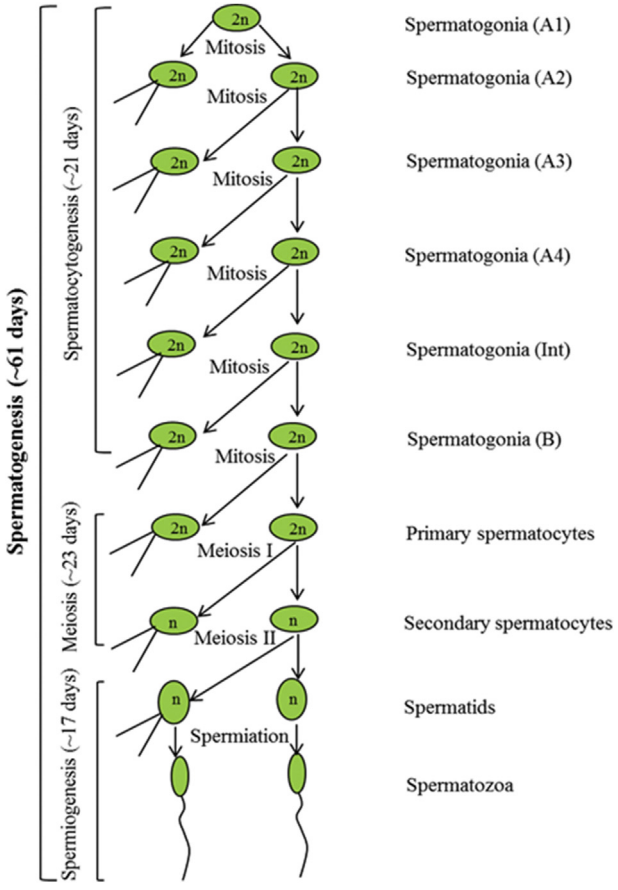

Fig. 2. Stages of spermatogenesis in bulls.

3.1. Epididymal maturation and storage of spermatozoa

Once formed within the seminiferous tubules, the immotile spermatozoa are released into the seminiferous tubular fluid and transported to the epididymis, where they gain the ability to move and fertilize the ova [61]. The epididymis is generally divided into three parts: caput, corpus, and cauda. In bulls, the transit of spermatozoa through the epididymis usually takes 8–11 days [62]. To attain the fertilizing capacity, spermatozoa undergo many maturational changes during their transit through the epididymal duct [61]. These include changes in plasma membrane lipids, proteins and glycosylation, alterations in the outer acrosomal membrane, gross morphological changes in the acrosome, and cross-linking of nuclear protamines, proteins of the outer dense fiber and fibrous sheath. The cauda or tail of the epididymis and proximal portion of the vas deferens are the regions where spermatozoa are stored before ejaculation [63,64]. During ejaculation, the stored spermatozoa and the surrounding fluid are mixed with the alkaline secretions of the male accessory sex glands, and the resulting so-called semen is deposited during mating into the vagina, cervix or uterus depending on the species.

3.2. Sperm capacitation and fertilization

Although spermatozoa acquire maturity during epididymal transit, they do not attain complete fertilizing capacity. To attain maximum fertilizing capability, spermatozoa must travel and reside in the female reproductive tract for a minimum period of time. During their stay in the female reproductive tract, spermatozoa undergo some biochemical changes that are known as sperm capacitation [65,66]. This includes the removal of a large portion of the extracellular coating proteins that prevent adhesion (including the decapacitation factors) during the transit of spermatozoa in the uterus [67]. The fertilization process involves a series of specific interactions between sperm and oocyte. Firstly, in the oviduct, the motility pattern of sperm becomes hyperactive which is considered to facilitate sperm-oocyte interaction. Secondly, some specific plasma membrane proteins overlying the acrosome are exposed and bind to the zona pellucida (ZP) of oocyte [68]. In mice, ZP comprises of three glycoproteins (ZP1, ZP2 and ZP3) while ZP3 acts as a primary sperm binding receptor [69]. In contrast, bovine ZP consists of ZP2, ZP3, and ZP4 while ZP3 alone cannot act as a sperm binding receptor, instead, a heterocomplex of ZP3 and ZP4 is responsible for sperm-zona binding [70]. Zona binding probably initiates acrosome reaction [61]. The acrosome reaction allows the release of a variety of enzymes mainly acrosin and hyaluronidase that hydrolyze zona proteins [71,72]. Subsequently, the mechanical force generated by the flagellar action of the tail pushes the spermatozoon to the perivitelline space of the oocyte. Then, the spermatozoon comes into the direct contact with the oolemma, the plasma membrane of the oocyte. At the moment of direct contact, sperm surface proteins, particularly Izumo [73], located in the

equatorial segment of the sperm head, are involved in adhesion and fusion with the plasma membrane of the oocyte [74]. Recently, it has been reported that during sperm and oocyte fusion, Izumo1 binds to a folate receptor termed Juno on the oocyte membrane and ultimately results in fertilization [75]. More recently, Izumo1 has also been identified in bovine spermatozoa and the aberrant location of Izumo1 in cryopreserved spermatozoa with damaged acrosomes, which have impaired fertilizing capacity in comparison with freshly ejaculated spermatozoa, indicates its possible role in fertilization [76]. Fusion of sperm and oocyte leads to depolarization of the oolemma which triggers the release of cortical granules located under the oocyte surface and results in blocking of polyspermia. Further, it has been reported that, after fertilization, Juno redistributed in the perivitelline space through extracellular vesicles which may bind and neutralize acrosome-reacted spermatozoa and possibly block polyspermy [77]. Soon after membrane fusion, the sperm nucleus starts to decondense which is needed to prepare the paternal chromosomes to pair with the maternal chromosomes. The decondensation of sperm nucleus starts through reducing disulfide bonds between protamines by a primary reducing agent, glutathione [78]. After decondensation of nuclear material, the paternal and maternal pronuclei are lined up to form a zygote [79]. However, the whole events including zona binding and penetration are highly dependent on the intact plasma membrane, acrosome, mitochondria as well as high quality nuclear material of a spermatozoon.

4. Effects of increased testicular temperature on semen quality

4.1. Ambient temperature effects and cattle breeds

Generally, the testes operate on the edge of hypoxia. Increased temperature increases testicular metabolism, with a concomitant higher need for oxygen to sustain the aerobic metabolism. However, the testicular blood flow increases very little in response to the increased testicular temperature and consequently the testes become more hypoxic [42]. As a result, the changes in sperm quality are clearly depicted by some older studies. Casadey et al. [80] exposed two *Bos taurus* bulls (Guernsey) to 37 °C with 81% relative humidity for 12 h per day for 17 consecutive days. The ejaculates were found to contain 30–40% morphologically abnormal spermatozoa (mostly coiled tails and detached heads) with a profound decrease in total sperm count, concentration, and motility. In another study, *Bos taurus* (Friesland) and *Bos indicus* (Afrikaner) bulls were exposed to ambient temperature at 40 °C with 35–45% relative humidity for as little as 12 h [81]. Although heat stress exposure to bulls was short, a significant decline in motility and percentage of live spermatozoa was observed in both breeds together with a significant increase in the percentage of morphologically abnormal spermatozoa. Moreover, *Bos taurus* bulls were found more susceptible to heat stress compared to *Bos indicus* bulls. Likewise, Johnston et al. [82] reported that crossbred (*Bos indicus* × *Bos taurus;* Brown Swiss and Red Sindhi) bulls exposed to high ambient temperature were comparatively less susceptible to heat stress in terms of semen quality and recovered more rapidly than purebred *Bos taurus* (Holstein) bulls.

Since then, several studies have investigated seasonal influences on semen quality in different breeds of bulls. Mathevon et al. [83] conducted a detailed study to evaluate seasonal influence on semen quality in 198 *Bos taurus* (Holstein) bulls during summer and winter seasons and observed that the summer season significantly affected all semen quality parameters. Furthermore, seasonal influence on semen quality was investigated in 6 *Bos taurus* (3 Limousin and 3 Simmental) and 5 *Bos indicus* (Nelore) bulls where *Bos taurus* bulls were found more heat susceptible in terms of producing high number of abnormal heads followed by cytoplasmic droplets throughout the study period [84]. In another study, Balic et al. [12] investigated seasonal influence on 19 *Bos taurus* (Simmental) bulls: they observed that the summer heat stress declined semen quality and they could relate this finding to the presence of high lipid peroxidation (as determined by thiobarbituric acid reactive substances; TBARS). Moreover, the authors observed that the semen collected from young bulls during summer season was associated with more intensive oxidative protein damage (assessed by protein carbonyl content) [12]. At the cellular level, heat stress exerts its adverse effects by directly unfolding and aggregating the proteins. It has also been reported that heat stress highly affects the synthesis of proteins followed by DNA and RNA (reviewed in Ref. [85]). Concomitantly, heat stress induces the activation of heat shock transcription factors (HSFs), mainly HSF1, which enables the production of heat shock proteins (HSPs) in order to reduce the detrimental effects of heat load (reviewed in Ref. [86]). If the heat stress continues, the production of HSPs is altered leading to changes in altered physiological state along with the increased production of reactive oxygen species (ROS) [85]. Likewise, in humans, defective semen samples have been shown to produce higher (40 times) amounts of reactive oxygen species (ROS) than semen samples with normal spermatozoa [87]. Reactive oxygen species are small, oxygen-based molecules that are highly reactive because of unpaired electrons. The most prominent ROS are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl ion (OH^-). Physiologically a certain amount of ROS are required for the regulation of several transmembrane signal transduction pathways in somatic cells [88] and for capacitation and acrosome reaction in spermatozoa [89]. In contrast, high concentrations of ROS reduce sperm motility and viability which culminate in poor sperm-zona pellucida binding [90]. Therefore, it is evident that increased temperature has an adverse effect on semen quality which might be related to the increased production of ROS. Failure to achieve this delicate balance of ROS in the seminal plasma may result in compromised field fertility.

4.2. Scrotal insulation as a model for in vivo heat stress study

Insulation of the scrotum by a special scrotal sac, made of nylon cloth with polyester batting has frequently been used as a model for mimicking increased testicular temperature. Scrotal insulation disrupts testicular thermoregulation by increasing the testicular temperature and also by interfering with scrotal sweating. Possibly the first study to evaluate the effects of scrotal insulation on sperm quality was conducted by Wildeus and Entwistle [91] in crossbred bulls (*Bos indicus* × *Bos taurus;* Holstein and Brahman). Morphological sperm abnormalities (mostly decapitated, protoplasmic droplets, and tail defects) appeared in the ejaculates in a chronological order depending on the stages of sperm maturation during heat insult, i.e. 48 h of scrotal insulation affected spermatozoa in the caput epididymis as well as spermatids in the maturation stage. Subsequently, Vogler et al. [62,92] conducted an elaborate study by insulating the scrotum of 6 Holstein bulls for 48 h. According to the reports, although the total number of spermatozoa was not significantly reduced, the proportion of progressively motile spermatozoa and the proportion of normal spermatozoa were decreased from 69% to 42% and 80% to 14%, respectively in the ejaculates collected after 15–18 days of scrotal insulation. The type and proportion of abnormal spermatozoa, and specific abnormalities appeared in a consistent chronological order although there was considerable variation in response to heat insult among bulls. In another study, Barth and Bowman [93] compared sperm abnormalities after scrotal insulation (4 days) of 4 mixed *Bos taurus*

bulls with sperm abnormalities after dexamethasone treatment for 7 days. Nuclear vacuoles, pyriform heads, and microcephalic spermatozoa appeared in the ejaculates collected 18–25 days post-treatment and these abnormalities were more prevalent in scrotal insulated bulls than in dexamethasone treated bulls. Conversely, dexamethasone treatment resulted in an earlier and more severe effect on epididymal spermatozoa. In a further study, Walters et al. [94] evaluated sperm abnormalities by insulating 4 Holstein bulls for 48 h and observed a high number of abnormal spermatozoa (81%) in semen collected after 27 days of scrotal insulation compared to the pre-scrotal insulation period (18%). The most important difference was observed in the percentage of pyriform shaped spermatozoa (51.6% vs 3.8%). Few years later, Fernandes et al. [95] examined sperm abnormalities by insulating scrotum of 4 zebu (Nelore) bulls for 5 days where head and chromatin defects were more prominent in the ejaculates collected at 14 and 21 days of post-insulation. Newton et al. [96] investigated sperm quality by insulating the scrotum (72 h) of 6 Holstein-Friesian bulls and observed thermal insult profoundly affected all sperm characteristics. The authors further reported a sequential appearance of specific morphological abnormalities throughout the post-insulation period but some abnormalities (detached heads, pyriform heads and midpiece defects) peaked at 15–24 days after scrotal insulation [96]. Therefore, the above studies clearly indicated that heat stress disrupts testicular thermoregulation which ultimately leads to various forms of sperm abnormalities that are summarized in Table 1. Importantly, sperm abnormalities appear in the ejaculates in a chronological nature depending on the severity of heat insult and seem to be related to stages of germ cell development during heat insult (Fig. 3). However, to our knowledge, no studies have yet been conducted to evaluate the exact stages of spermatogenesis which are vulnerable to heat stress by collecting ejaculates throughout the spermatogenic cycle. In order to address the above mentioned questions we, therefore, artificially increased the testicular temperature by scrotal insulation of two breeds of bulls (Holstein Friesian and Belgian Blue) for 48 h. We observed that germ cells at meiosis and spermiogenetic stages of development were more susceptible to heat stress. Later, we could relate the possible causes of cell vulnerability of such specific periods to inefficient replacement of histones by protamines, leading to altered sperm chromatin conformation [17].

5. Heat stress and sperm epigenetics

The growing evidence supports the notion that certain paternally acquired traits as a result of ancestral exposures to mental stresses or diet changes can be inherited to the offspring as sperm epigenetic memory [97]. This is may be due to the special orchestration of the sperm nucleus, possibly helps in escaping from epigenetic reprogramming. The DNA in the sperm nucleus is uniquely arranged to meet the needs of this highly specialized cell. The unique nuclear protein landscape in a spermatozoon makes a chromatin structure that is between 6 and 20 times more compact than nucleosome-bound DNA, resulting in a tightly condensed nucleus [59,60,98]. Replacement of histone-bound chromatin to protamine-bound chromatin is responsible for such high compaction, necessary for the safe delivery of sperm DNA to the oocyte through protecting oxidative stress in the female reproductive tract (Fig. 3). Moreover, the highly compacted sperm nucleus blocks the transcriptional activity of sperm DNA. A lack of protamines in the sperm nucleus leads to DNA damage and could potentially cause male subfertility or infertility [99–101]. Since protamination is such an important aspect of sperm chromatin condensation, it was a major finding that heat stress affects sperm remodeling procedures (protamine-DNA compaction), which resulted in a higher incidence of protamine deficiency or loosely protaminated spermatozoa in heat-stressed ejaculates. In this regard, we assessed spermatozoa for protamine deficiency in semen collected after scrotal insulation using the Chromomycin A_3 (CMA_3) staining. This probe binds in the minor groove of GC-rich DNA in the absence of protamine that serves as a marker for the efficiency of DNA protamination [102,103]. In our study, we observed an increase in protamine deficient spermatozoa especially in the population of spermatozoa which were at the spermiogenic and meiotic stages of development at the moment of scrotal insult [17]. Therefore, it is postulated that heat stress at the spermiogenic and meiotic stages causes defective chromatin protamination by affecting mainly during the period of transition of histones to protamines (Fig. 4).

Likewise, environmental stresses have been reported to make changes in the DNA methylation pattern of spermatozoa [104]. DNA methylation denotes addition of a methyl group on the 5 carbon of cytosine residues (5 mC) at cytosine-phosphate-guanine dinucleotides (CpGs), which exert strong epigenetic regulation in many cell types. Studies have shown that DNA methylation is essential in genomic imprinting, gene expression regulation, X chromosomal inactivation, and embryonic development [105,106]. This epigenetic marker (5 mC) can activate or repress gene transcription at specific sites based on the methylation levels at promoter regions. Generally promoter regions are associated with CpG islands, where they remain hypomethylated and thus allow gene expression [107,108]. On the other hand, when a CpG island in the promoter region becomes hypermethylated, the expression of the gene is repressed [108–110].

The significance of DNA methylation has been demonstrated globally, locally, and at the single locus level in both humans and animal models. Several studies demonstrated that aberrant methylation in genes at promoter and imprinted loci are strongly associated with various forms of infertility and sperm defects in

Table 1
Major sperm morphological abnormalities observed in several studies after inducing heat stress using scrotal insulation (SI).

Duration of SI	Time in days at which sperm morphological abnormalities appear in the ejaculates after SI	Major sperm morphological abnormalities	References
48 h	6-23 days	Decapitated sperm, abnormal acrosomes, abnormal tails and protoplasmic droplets	Wildeus and Entwhistle [91]
48 h	12-36 days	Tailless sperm, diadem defects, pyriform head, nuclear vacuoles, knobbed acrosomes and drag defects	Vogler et al. [62,92]
96 h	18-25 days	Pyriform heads, nuclear vacuoles, microcephalic sperm and abnormal DNA condensation	Barth and Bowman [93]
48 h	23-34 days	Pyriform heads, diadem defects, apical vacuoles	Walters et al. [94]
120 h	14-21 days	Head abnormalities, nuclear vacuoles, acrosome and midpiece defects	Fernandes et al. [95]
72 h	15-49 days	Pyriform heads, detached heads, midpiece defects, proximal droplets	Newton et al. [96]
48 h	14-42 days	Pyriform heads, large heads, nuclear vacuoles	Rahman et al. [17]

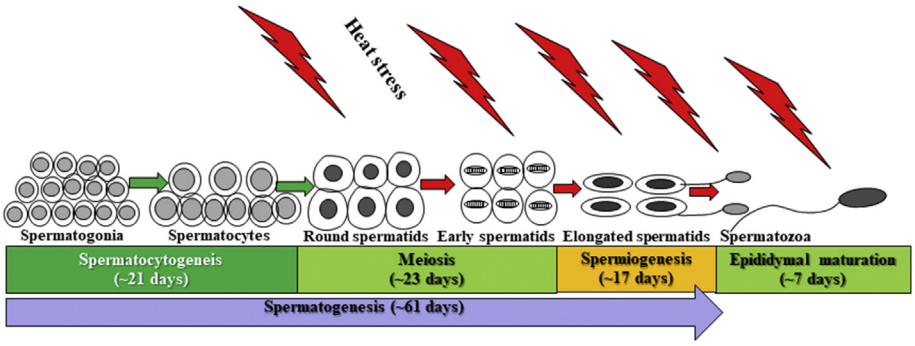

Fig. 3. Several stages of bovine spermatogenesis are vulnerable to heat stress. The figure is illustrated based on the literature data that are presented in Table 1.

men [111,112]. Likewise, global sperm DNA hypomethylation was related to poor pregnancy outcomes in IVF patients [113]. There are many likely candidates that may cause epigenetic alterations in spermatozoa and may lead to abnormal embryogenesis [114,115]. Environmental toxins and aging have been shown to be important for altered sperm epigenome elsewhere [116,117]. Alterations of methylation patterns on the paternal genome have been studied in several experiments by using 5-azacytidine and 5-aza-2′deoxycytidine. These are the potent DNA methylation inhibitors and have commonly been used for methylation studies [118]. Short-term exposure of both rats and mice to these inhibitors has induced a sharp decline in fertility whereas long-term exposure resulted in more severe phenotypes as well as embryo lethality [119]. Further studies also reported almost the similar effects of reduced fertility and preimplantation loss while mice were treated with 5-aza-2′deoxycytidine [120,121]. Hence, it is clear that the use of DNA methylation inhibitors can reduce global methylation patterns of the germ cells that may, at least in part, be responsible for abnormal embryogenesis.

A key process in the acquisition of nuclear totipotency of mammalian zygotes is the erasure of epigenetic marks acquired immediately after fertilization. In bovine zygotes, it has been shown that cytosine in the paternal pronucleus is actively and almost completely demethylated followed by *de novo* methylation before the two-cell stage while the levels of cytosine methylation gradually decrease in the maternal pronucleus [23–25] (Fig. 5). This dynamic DNA methylation reprogramming within the narrow developmental window of the pronuclear stage is very important for normal embryogenesis. Therefore, it has been postulated that heat stress during spermatogenesis alters sperm chromatin conformation, which may ultimately perturb the dynamics of the DNA methylation reprogramming in the zygote [24]. According to our hypothesis, we observed reduced fertilization rates and perturbed DNA methylation reprogramming in the paternal pronucleus following *in vitro* fertilization of oocytes with heat-stressed spermatozoa [24]. However, whether the alterations of sperm methylation are solely responsible for reduced fertilization rates and perturbed methylation reprogramming in the paternal genome, a genome-wide methylation study of the spermatozoa is needed.

6. microRNA populations and sperm epigenetic regulation

The possible role of sperm RNA transcripts in epigenetic regulation has been discussed in a review paper by Jenkins and Carrell [122]. Recent evidence suggests that the RNA transcripts involved in epigenetic regulation are belonging to the family of small non-coding RNAs (18–24 nt), mostly microRNAs (miRNAs). These miRNAs along with small interfering RNAs (siRNAs) regulate gene expression either by inhibiting or activating translation or targeting mRNAs for degradation usually by binding to a 3′UTR [123]. Some miRNAs act as transcriptional regulators by targeting other regions of gene such as promoter [124,125]. It is possible that in the transcriptionally quiescent spermatozoa, miRNAs provide a signal for early embryonic histone replacement [60], which is necessary for DNA methylation reprogramming in the early developing embryos. The most abundant miRNA in human spermatozoa is miR-34c [126]. Subsequently, it has also been identified in spermatozoa of stallion, mouse and bull [127–129]. With regard to importance of miR-34c, Liu et al. [130] showed that it is essential for the first cleavage division in mouse zygotes. However, its mechanism of action and the functional role in spermatogenesis or fertility is not fully known. Since the miR-34c is sperm-borne and is required for the first cleavage division in mouse, we postulate that it can be used

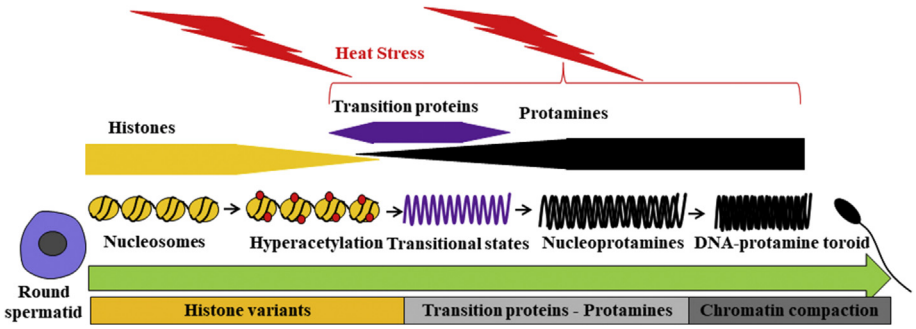

Fig. 4. Germ cell remodeling events: the heat stress vulnerability. The extensive incorporation of histone modifications and global hyperacetylations makes open chromatin domains containing unstable nucleosomes which are replaced primarily by transition proteins and later by protamines. The unstable forms of nucleosomes are vulnerable to heat stress and consequently DNA-protamine compaction is affected.

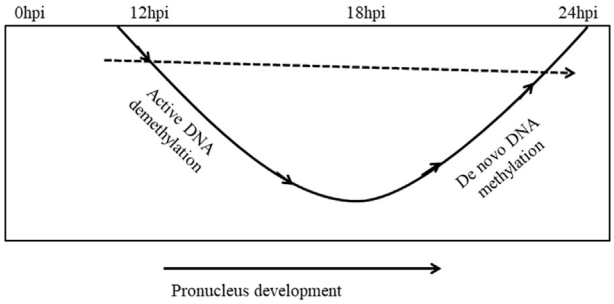

Fig. 5. Schematic presentation of DNA methylation dynamics in the bovine male (solid line) and female (dashed line) pronuclei at hour post insemination (psi) of spermatozoon [24].

as a biomarker for bull fertility. Somewhat in line with this hypothesis, sperm miRNA expression between high and low fertility bulls was investigated through microarray-based study [131]. The authors reported that seven miRNAs (hsa-aga-3155, -8197, -6727, -11796, -14189, -6125, -13659) were differentially expressed between high versus low fertility bulls [131]. However, miR-34c was not in the differentially expressed miRNAs, possibly due to a number of limitations including individual variations of bulls, semen collection times, nutrition condition or animal health or technical variations and most importantly microarray contained probes that were known miRNAs for humans, mice and rats. In human, miRNAs (miR-26a, -299a, -216, and -234b-3p) have been shown as differentially expressed in abnormal versus normal spermatozoa [132]. Likewise, in pigs, let-7 and miR-22 have been found as differentially expressed in abnormal sperm morphology [133]. Further studies reported that many specific miRNA may come forward to help cell's function in stressed conditions. In environmental stress, the most important player is p53, a tumor suppressor, which is activated upon DNA damage and regulates the expression of miRNAs (miR-34a, miR-34b and miR-34c) [133,134]. These miRNAs are, in fact, found as repressors of a number of shared target genes in promoting cell growth arrest or apoptosis. Importantly, potential epigenetic mechanisms involved in paternal stress contribution to offspring have been tested by exposing mice to 6 weeks of chronic stress before breeding [135]. The authors observed 9 specific miRNAs (miR-193-5p, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, miR-532−3p, and miR-698) that have significantly been increased in spermatozoa that have an impact on offspring's responses to stress [135]. Recently, Nehammer et al. [136] conducted a study where they exposed specific miRNA mutant *Caenorhabditis elegans* to heat stress at 32 °C for 15, 20, and 25 h and compared the ability to survive the heat stress. The research group reported that deletion of miR-71, miR-80, miR-229, and the miR-64-66 clusters resulted in heat sensitivity while deletion of miR-239 resulted in heat resistance. However, to date, no information is available on whether heat stress in bulls can have an impact on sperm miRNA-induced epigenetic regulation. Considering the susceptibility of miRNAs to the environmental changes, it is postulated that heat stress in bulls may have a great impact on sperm miRNA populations which are responsible for epigenetic regulation. Therefore, the possibility of using miR-34c as a biomarker for bull fertility either in normal or stressed conditions needs more investigations possibly by using the robust technique, miRNA-seq.

7. Conclusions and future directions

In light of the preceding discussion, it has become obvious that heat stress can have severe effects on bull's fertility, and the severity of the effects may be related to the bull's breed. If heat stress affects sperm quality it may exert a subtle effect that remains largely unnoticed by conventional semen analysis but which may have prolonged effects on subsequent field fertility. Our *in vivo* heat stress study signifies that heat stress can induce changes in sperm chromatin conformation, which lead to sperm abnormalities and low fertility. The data can practically be implemented to avoid wrongful culling of young bulls by commercial semen producers. This culling is often based on the increased presence of morphologically abnormal spermatozoa which might be due to heat stress exposure. In commercial AI centers, young bulls (~11 months old) are culled when their ejaculates have less than 75% normal spermatozoa [137]. If these morphologically abnormal spermatozoa are due to heat stress, then the wrongful culling of these young bulls will lead to higher economic losses. There is a higher chance of wrongful culling of young Belgian Blue bulls since this breed is more susceptible to heat stress. Although a few published reports are available, this problem may happen in tropical countries since the bulls are more susceptible to suffer from heat stress. When heat stress is suspected even for a shorter period of time, a prolonged testing period for young bulls should be considered to prevent unreasonable and early culling of valuable bulls. This will optimize profitability of the breeding station, notwithstanding the extra costs for the prolonged testing period. Supplementation of bull ration with commercially available 100−160 g Omega-3 daily for each bull can be a mitigation option to some extent under heat-stressed environments [138] as it has also been reported to protect sperm membrane during cold shock or cryopreservation (reviewed in Ref. [139]). When the heat stress cannot be avoided, even by proper cooling systems in the stable of breeding bulls located especially in tropical or subtropical countries, stopping of semen collection until it cools down would be a practical solution. Identification of heat stress-induced up- or down-regulation of specific miRNAs that are responsible for post-transcriptional regulation of mRNAs essential for early embryo development can be a future prospective field of research in order to restore bovine fertility potential and as such may be equally applicable for humans.

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