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Exploring the Effects of Nitric Oxide and Reactive Oxygen Species on Buffalo Sperm Quality: A Comprehensive Review

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Buffalo reproduction plays a vital role in the livestock industry, where sperm quality is a critical determinant of successful fertilization and overall fertility. Nitric oxide (NO) and reactive oxygen species (ROS) are key regulators of various physiological processes, including sperm function. However, their dual role in modulating sperm quality is complex, as both excessive and insufficient levels can lead to detrimental effects. This review explores the intricate balance between NO, ROS and sperm quality in buffalo, emphasizing the molecular mechanisms underlying these interactions. NO is a signaling molecule involved in the regulation of sperm motility, capacitation and acrosome reaction, but its overproduction can result in nitrosative stress, impairing sperm function. Similarly, ROS, at physiological levels, are essential for sperm capacitation and acrosome reaction, yet an imbalance towards oxidative stress can lead to lipid peroxidation, DNA fragmentation and reduced sperm viability. This review also delves into the role of exogenous NO donors, such as sodium nitroprusside (SNP), in modulating NO and ROS levels, and their consequent impact on sperm quality. The dual effects of NO and ROS on buffalo sperm highlight the importance of maintaining an optimal redox balance to preserve sperm integrity and functionality. In conclusion, understanding the molecular pathways through which NO and ROS influence sperm quality is crucial for developing targeted strategies to enhance reproductive outcomes in buffalo. Future research should focus on identifying biomarkers of oxidative stress and evaluating the potential of antioxidant therapies to mitigate the adverse effects of NO and ROS, ultimately improving buffalo fertility.

Keywords: Buffalo; nitric oxide; ROS; SNP; sperm.

1. INTRODUCTION

Indian buffalo breeds are among the finest globally, with nearly half of the world's buffalo population found in India. High-quality semen is crucial for ensuring fertility in bovines, as sperm quality is closely linked to reproductive success [1]. To maximize productivity in buffaloes, it is essential that the majority of females are inseminated with semen from bulls of superior genetic quality. Consequently, buffalo bulls are maintained in numerous centers across the country to facilitate widespread use of artificial insemination (AI) in this species.

Buffalo biotechnology is gaining importance as this species is unique to our country. However, the procedures involved in these technology need to be perfected [2]. Decline in the reproductive performance of buffalo poses demands to sort out the factors that contribute fertility. A number of factors, including sperm capacitation, confer the fertilizing ability to sperm are found to be responsible for altering the fertility.

"The surface of buffalo spermatozoa is heavily glycosylated due to the presence of glycoproteins and Glycosylphosphatidylinositol-anchored proteins. These glycoproteins have immune-regulatory and reproduction-specific functions. Buffalo spermatozoa contain many proteins, including those involved in glycolytic processes, mitochondrial respiratory chain, protein folding and spermatogenesis. Transcriptomic profiling of buffalo spermatozoa detected some transcripts, which were associated with biological processes, molecular function and cellular components. In recent years, male fertility markers have been extensively studied to understand the molecular mechanisms leading to subfertility and to develop accurate diagnostic and therapeutic strategies. One of the most promising areas of research is the effect of oxidative stress (OS) in semen. OS can be defined as the imbalance between pro-oxidative and anti-oxidative molecules in a biological system which arises as a consequence of excessive production of free radicals and impaired antioxidant defense mechanism" [3,4,5].

"Free radicals derived from oxygen, known as reactive oxygen species (ROS), include superoxide ($O2^-$), hydrogen peroxide (H_2O_2), peroxyl (ROO-), and hydroxyl (OH) radicals" [6]. "Nitrogen-derived free radicals, referred to as reactive nitrogen species (RNS), include nitric oxide (NO-), nitrogen dioxide (NO_2), and peroxynitrite anion (ONOO-)" [7,8]. RNS are often considered a subclass of ROS [9]. Depending on their concentration and nature, these free radicals can have either beneficial or detrimental effects on sperm function [10]. "ROS such as hydrogen peroxide, superoxide anion, and hydroxyl radicals are known to affect various functional sperm parameters" [11].

"Nitric oxide (NO) has recently gained attention as a crucial intercellular and intracellular messenger involved in various physiological processes. NO is synthesized from L-arginine by nitric oxide synthase (NOS), an enzyme that exists in three isoforms. Two of these isoforms-endothelial NOS (eNOS) and neuronal NOS (nNOS)-are collectively known as constitutive NOS (cNOS), responsible for the continuous basal release of NO and requiring calcium/calmodulin for activation. The third isoform, inducible NOS (iNOS), is responsible for the prolonged release of NO and does not require calcium/calmodulin for activation, being expressed in response to inflammatory cytokines and lipopolysaccharides" [12].

"While NO is essential for maintaining sperm motility, excessive levels can be toxic" [13]. "Other RNS, such as nitrogen dioxide radical and peroxynitrite anion, are also damaging to sperm cells. The primary mechanism of NO-induced sperm damage likely involves the inhibition of mitochondrial respiration and DNA synthesis" [14]. "NO toxicity is also mediated indirectly through its interaction with superoxide anions, leading to the formation of peroxynitrite anion. When protonated, peroxynitrite decomposes to form hydroxyl and nitrogen dioxide radicals, both of which are highly cytotoxic" [15].

In buffaloes, low concentrations of NO have been found to increase sperm motility and viability, whereas high concentrations have the opposite effect [16]. In vitro capacitation of spermatozoa, a critical step in in vitro embryo production (IVEP), can be enhanced by supplementing with additives such as heparin, albumin, and various antioxidants. Recently, NO has emerged as a potent regulator of sperm functions, highlighting its potential role in improving reproductive outcomes in buffaloes.

2. EFFECT OF NITRIC OXIDE ON MALE REPRODUCTION

"Nitric oxide (NO) plays a critical role in various aspects of male reproduction. Francavilla et al. demonstrated that NO is produced in spermatozoa through the action of constitutive nitric oxide synthase (cNOS)" [17]. This NO generation is involved in the fusion of sperm with the oocyte, although it does not play a role in zona pellucida binding. Herrero et al. highlighted the significance of sodium nitroprusside (SNP), a compound that releases NO, as a key signaling molecule in mammalian sperm functions, including motility, capacitation, and the acrosomal reaction [18]. Thaler and Epel further observed that NO exerts a concentration-dependent effect on reproductive processes [19]. Specifically, they found that a narrow, typically low, range of NO concentration enhances the early events of reproduction. However, both the absence and excess of NO can have detrimental effects. They proposed that the widespread presence of NO signaling in animals likely controls reproduction more effectively through its target mediators rather than through nitric oxide synthase (NOS) enzymes directly. Cristian et al. suggested that NO, produced by sperm NOS in cryopreserved bovine semen, is actively involved in sperm capacitation and the acrosomal reaction, further underscoring the importance of NO in male reproductive physiology [20].

3. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM MOTILITY AND SPERM VIABILITY

High levels of ROS (superoxide, hydrogen peroxide and hydroxyl radical) endanger sperm motility, viability and other functions by interacting with membrane lipids, proteins and nuclear and mitochondrial DNA [21] with direct effect on intracellular Ca^{++} level by promoting Ca^{++} sequestration inside the mitochondria [22]. ATP generated by mitochondrial activity is required for sperm motility [23]. Increased level of ROS showed adverse effect on sperm motility, viability, and membrane lipids [24]. Kovalski et al. showed that hydrogen peroxide and hydroxyl radical but not the superoxide radical inhibited the motility and viability of human spermatozoa [25].

Aitken et al. reported that a low concentration of hydrogen peroxide did not have any effect on sperm motility, but suppressed sperm-egg fusion. But high concentration affected the motility and viability of spermatozoa [26].

Oxidative stress may cause functional changes in sperm motility, viability and other functions. Furthermore, dealing with infertility problems may also significantly contribute to chronic stress in a subject which in turn may influence general health [27]. Agarwal et al. stated that high ROS level affects the spermatozoa concentration, motility and morphology [8]. Kao et al. suggested that oxidative stress impairs sperm motility and results in less number of sperm reaching the oocyte for fertilization [28]. "ROS damage the

sperm membrane, which in turn reduce the sperm motility, viability, ability to fuse with the oocyte and directly damage sperm DNA" [29].

4. EFFECT OF NITRIC OXIDE ON SPERM MOTILITY AND SPERM VIABILITY

Keller and Polakoski observed that L-arginine, the substrate for NOS enhances sperm motility *in vitro* [30]. Supplementation with L-arginine stimulates the motility of rabbit and human spermatozoa *in vitro* [31]. NO increased cGMP level. cGMP was involved in the metabolic activity and had been shown to increase sperm metabolic rate [32]. Higher concentration of SNP (1-100 µM) caused a decrease in sperm motility without affecting sperm viability [33]. Herrero et al. suggested that the *in vitro* studies have shown that low concentrations of NO enhance the motility of mouse spermatozoa [34].

Weinberg et al. suggested that there is "involvement of NO in sperm fertilizing ability and lower concentration of NO results in a significant increase in sperm capacitation, although there are conflicting reports concerning the effects of the NO on the sperm motility and viability" [35]. "Higher NO concentrations seem to exert opposite effects on the motility, viability and metabolism of human spermatozoa *in vitro"* [36]. "High NO levels have been found in semen of infertile man with decreased sperm motility and inhibitors of NOS have enhanced the motility, whereas addition of SNP to spermatozoa of infertile patients further diminished motility, which may be due to high level of OS" [37]. Nobunaga et al. noticed "a negative correlation between the concentrations of NO and the percentage of motile cells and suggested that lower concentrations of NO enhanced the motility in human" [38].

Revelli et al. reported that "NO has been recently shown to modulate *in vitro* motility, viability, acrosomal reaction and metabolism of spermatozoa" [39]. NO were inversely correlated with sperm motility, linearity and amplitude of sperm lateral movement, viability, membrane function and nuclear DNA integrity [40]. Khodaei and Hejazi noticed that "low concentration of NO increased the motility and viability of spermatozoa. However, high concentration of NO decreased the sperm motility and viability in ram" [41]. Vidya et al. suggested that more concentrations of NO affect sperm parameters such assperm concentration, sperm motility, viability and sperm morphology [42].

5. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM MORPHOLOGY

Menkveld et al. stated that ROS production causes abnormalities of spermatozoa i.e. globozoospermia, flagellar abnormalities, large headed, multiple tailed and elongated head spermatozoa [43]. "Abnormal sperm morphology, specifically midpiece abnormality, is associated with elevated levels of ROS" [44]. "The capacity for ROS generation is significantly enhanced in abnormal spermatozoa. ROS have been linked to abnormal sperm morphology" [45].

"Canine spermatozoa with significant diminution of motility, plasma membrane functionality and greater percentage of abnormalities produced greater amount of ROS as compared with normal spermatozoa" [46]. "GilGuzman showed that levels of ROS production were negatively correlated with teratozoospermia and spermatozoa developmental stages" [47]. "OS due to excessive generation of ROS is presumed to cause spermatozoa DNA damage, sperm abnormalities and has correlated positively with apoptosis" [48]. Aziz et al. reported that ROS level was negatively correlated with normal morphology and borderline morphology and was positively correlated with proportion of sperm with amorphous heads, damaged acrosomes, midpiece defects, cytoplasmic droplets, tail defects and sperm deformity index (SDI) scores [49].

6. EFFECT OF NITRIC OXIDE ON SPERM MORPHOLOGY

Ambrosini et al. demonstrated that abnormal spermatozoa were increased in presence of high concentration of NO [50]. Huang et al. stated that the production of NO was found to selectively affect sperm morphology in human [51]. Several studies reported that negative correlation existed between NO levels and sperm motility, morphology and DNA fragmentation [52,53,54]. "Spermatozoa susceptible to ROS attack result in decreased sperm motility by rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased midpiece sperm morphological abnormalities" [55,56,5].

Vidya et al. showed that "possible role of NO in male infertility. They found a negative correlation between NO concentrations and sperm

morphology" [57]. Ramya et al. suggested that high levels of NO result in abnormalities in the spermatozoa [58].

7. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM FUNCTIONAL MEMBRANE INTEGRITY

"The peroxides constitute a potential hazard to the structural and functional integrity of spermatozoa, lessening the metabolic activity of cells that are aging either within the reproductive tract *in vivo* or during storage *in vitro"* [59]. Sperm function test was carried out by hypo-osmotic swelling test i.e. functional membrane integrity was affected by increased concentration of ROS in the spermatozoa [60]. "ROS act on cytosolic or membrane compounds such as lipids or proteins with thiol groups and affecting the integrity of the sperm membrane" [61].

"Unsaturated fatty acids give the sperm plasma membrane fluidity, which needs to participate in the membrane fusion events associated with fertilization. These molecules are also vulnerable to attack by ROS initiating LPO cascade that can seriously compromise the functional membrane integrity of sperm cells" [62]. Twigg et al. demonstrated that endogenous as well as exogenous ROS can be detrimental to functional membrane integrity of spermatozoa [63]. High concentrations of ROS induced loss of motility which lead to sperm dysfunctions and affect sperm functional membrane integrity at different levels [64]. "Highly toxic metals in humans and other mammals can induce OS through their capacity to interact with ROS, increasing their oxidant activity or by affecting membrane integrity" [65].

8. EFFECT OF NITRIC OXIDE ON SPERM FUNCTIONAL MEMBRANE INTEGRITY

Chamberland et al. suggested that high dose of NO affect functional membrane integrity, vigor and capacitation of spermatozoa [66]. Srivastava et al. reported that NO pathway provides an important antioxidant action that protects cells from lipid peroxidation (LPO) i.e. by reaction of free radicals with lipids in cell membranes resulting in destruction/modification of numerous lipid molecules and leading to loss of the sperm membrane integrity [67]. Ferrusola et al. reported that increase in NO production causes decreased functional membrane integrity in stallion spermatozoa [68]. Leal et al. stated that addition of 10 mM L-arginine to the capacitation medium increased NO synthesis, sperm progressive motility, vigor and functional membrane integrity in bull semen [69]. Gundogan et al. reported that the increased concentration of NO affects functional membrane integrity and DNA integrity of ram semen [70].

9. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM ACROSOMAL INTEGRITY

Aitken and Fisher reported that ROS in male reproductive tract have become a real concern because of their potential toxic effects at high levels on sperm quality i.e. acrosomal integrity [71]. The decrease in sperm motility associated with ROS occurs in the absence of any detectable decrease in viability, acrosomal integrity or mitochondrial membrane potential or any detectable increase in LPO [72]. Ball et al. suggested that OS resulted in rapid loss of acrosomal integrity of equine spermatozoa [73]. Acrosome integrity and plasma membrane integrity were decreased by increasing the production of ROS [74].

10. EFFECT OF NITRIC OXIDE ON SPERM ACROSOMAL INTEGRITY

Herrero et al. speculated that acrosomal reaction promoted Ca^{++} influx which activated sperm NOS (calcium dependent form) leading to NO synthesis which might interact with enzymes involved in different signal transduction pathways that finally resulted in acrosomal exocytosis [75]. Gye reported that SNP, a NO generating agent increased spontaneous acrosomal reaction in mouse [76]. Herrero et al. reported that acrosome reaction inducing effect of NO-releasing compounds occurred via an increase in cGMP levels and protein kinase G activation [18].

Reyes et al. observed that NOS activity in bovine sperm was first appeared in the acrosome, then 60 minutes later in the head, middle piece, cytoplasmic droplet and tail. [77]. Rodriguez et al. noticed that NO induced acrosome reaction in capacitated spermatozoa involving hydrogen peroxide with the participation of protein kinase A (PKA), protein kinase C (PKC) and protein tyrosine kinase as part of the signal transduction mechanism in cryopreserved bovine spermatozoa [78].

11. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

ROS formation results in the decreased sperm MMP and motility. Since, high sperm MMP is required for mitochondrial adenosine triphosphate (ATP) production and sperm motility [11]. Kao et al. suggested that sperm MMP is the major target of attack by exogenous ROS [79]. Armstrong et al. reported that inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP with a significant decline in the MMP without any elevation in LPO [80]. Gravance et al. suggested that reduction in sperm motility may be due to an impairment of mitochondrial activity [81]. Vernet et al. suggested that ROS generated by the spermatozoa decreases MMP in rat epididymal spermatozoa [82]. Marchetti et al. suggested that the production of ROS causes the decreased MMP of spermatozoa [83]. Henkel et al. stated that DNA fragmentation is preceded by severe disruption in mitochondrial function detected as a decrease in MMP and this reduction is accompanied by the production of ROS [84]. ROS formation may be related to a decrease in MMP below a critical threshold [85]. ROS lead to oxidative damages which are numerous, ranging from membrane damage, inhibition of respiration, leakage of intracellular enzymes, axonemal protein damage and MMP damage of spermatozoa [86]. The production of potentially harmful ROS may attack on MMP [87].

Koppers et al. showed that the source of ROS is responsible for inducing oxidative damage in human spermatozoa which has implicated the mitochondrial function [88]. Guthrie et al. suggested that reduction in MMP might be due to oxidative damage by the formation of ROS and subsequent membrane lipid peroxidation (LPO) [89]. ROS could make a significant contribution to the induction of OS and DNA damage in spermatozoa and reduces the MMP of spermatozoa [90]. ROS production caused MMP defects. These are known factors for physiological dysfunctions including infertility [91].

12. EFFECT OF NITRIC OXIDE ON SPERM MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

RNS such as NO, nitrogen dioxide and peroxynitrite anion are considered to be damaging to sperm cells. The primary mechanism of NO induced sperm damage is likely to be the inhibition of MMP and DNA synthesis [92]. NO induced toxicity is also mediated indirectly through its interaction with superoxide and formation of peroxynitrite which causes the decreased in MMP [93]. The simultaneous formation of NO and superoxide produces peroxynitrite, a very strong oxidant and nitrating agent. This is capable of inhibiting important mitochondrial enzymes and affecting MMP of spermatozoa [94]. NO affects the mitochondrial function by interaction with low molecular weight thiols such as glutathione and protein thiols [95]. NO regulates the mitochondrial function by binding to cytochrome c oxidase, the terminal enzyme in the electron transport chain. It competes with oxygen, inhibiting the activity of the enzyme and thus negatively regulating mitochondrial oxidative phosphorylation particularly at the low oxygen concentrations in the spermatozoa [96].

13. EFFECT OF REACTIVE OXYGEN SPECIES ON SPERM LIPID PEROXIDATION (LPO) STATUS

The higher level of hydroxyl radicals results in the powerful initiators of LPO and would be expected to impair human sperm function through LPO induced changes in membrane fluidity and integrity [97]. Foote et al. suggested that the oxidizing ability greatly increased LPO status of spermatozoa [98]. Hyslop et al. suggested that hydrogen peroxide causes perturbations of important biochemical functions including increased formation of oxidized intracellular sulfhydryls, rapid decrease in ATP levels and a consequent depression of glycolytic flux. These processes occur before loss of plasma membrane integrity or increased LPO [99]. Aitken et al. showed that LPO is significantly accelerated in populations of defective spermatozoa exhibiting high levels of ROS production [100].

The ROS level in frozen-thawed spermatozoa is comparable with fresh spermatozoa. Significantly, higher LPO in frozen-thawed sperm compared to fresh sperm is observed. This may be due to the fact that the frozen-thawed bull sperm are more easily peroxidized than fresh sperm [101]. deLamirande and Gagnon stated that motility is impaired because of ATP depletion during LPO of the sperm plasma membrane and LPO increased in proportion to a decrease in superoxide dismutase (SOD) [102].

Alvarez and Storey reported that ROS causes increased LPO of sperm plasma membrane resulting in alteration of sperm function and DNA fragmentation in ejaculated spermatozoa [103]. LPO of sperm membrane is generally considered as the first marking point of sperm cell damage induced by reactive oxygen intermediates, which in turn may lead to sperm dysfunction that results in the inability of sperm to penetrate the oocyte [104]. Agarwal et al. stated that the high concentration of ROS especially in sperm plasma membrane causes increased LPO [105]. The action of ROS on human spermatozoa results in decreased capacity for ionophore induced acrosome reaction, decrease in sperm motility and increase in the concentration of LPO status [106]. Sikka suggested that a relationship exists between an increase in ROS induced OS, LPO, decreased level of SOD and motility in spermatozoa [107]. LPO level is higher in frozen-thawed sperm due to aromatic amino acid oxidase enzyme activity in dead sperm [108].

Peroxidative damage initiated by high ROS generation during OS as seen in spermatozoa of infertile men is associated with a loss of membrane function and damage to the DNA located in sperm head and increases LPO status of spermatozoa [109]. ROS production in sperm suspension, LPO and DNA oxidation are associated with poor sperm functions and subfertility [110]. Spermatozoa lose their motility more rapidly when incubated with oxygen. Due to high content of polyunsaturated fatty acids, the sperm membranes are prone to LPO [111]. ROS have detrimental effects on the peroxidation of membrane lipids leading to compromised sperm-oocyte fusion and decreased chromatin quality [112]. ROS is associated with cell damage including morphological defects, DNA fragmentation, LPO and impaired fertilization ability of spermatozoa [113]. One of the biomarkers of OS is malondialdehyde (MDA), an end product of LPO. The acyl chains of decosahexaenoic acid (DHA) bound to phospholipids of sperm membrane are particularly susceptible to ROS attack leading to the formation of MDA [114].

14. EFFECT OF NITRIC OXIDE ON SPERM LIPID PEROXIDATION (LPO) STATUS

NO may react with superoxide or hydrogen peroxide, resulting in the formation of peroxynitrite, hydroxyl radical, nitrogen dioxide or singlet oxygen, which cause oxidation of sperm membrane lipids and thiol proteins [115]. Despite being a free radical itself, NO can act as a free radical scavenger at low concentration, inactivating and even inhibiting production of superoxide which cause LPO, a process which leads to functional impairment of spermatozoa [116]. Hellstrom et al. showed that exogenous treatment of human spermatozoa with SNP was associated with enhanced post-thaw motility and viability while reducing lipid peroxidative damage to cellular membranes [117]. Darley Usmar and Halliwell reported that at micromolar concentrations, NO reacts with superoxide to generate peroxynitrite, which leads to iron sulphur cluster destruction, LPO, thiol nitrosylation and amino acid residue oxidation/nitration [118]. Watson suggested that toxic effect of NO on biochemical damage such as LPO, premature ageing and phase membrane transitions have been identified as important factors in sub-lethal damage of the spermatozoa that survive freezing and thawing [119]. Amongst ROS and RNS, peroxynitrite resulting from the diffusion controlled reaction of NO and superoxide and peroxyl radicals resulting from LPO radical chain reactions are important cellular components of oxidative stress [120,121]. High concentrations of NO react with superoxide result in the formation of peroxynitrite which causes LPO of sperm plasma membrane lipids [122].

15. CONCLUSION

The review highlights the critical role of reactive oxygen species (ROS) and nitric oxide (NO) in regulating sperm function, particularly in buffaloes, which are of significant importance to the Indian livestock industry. While ROS and NO are essential in maintaining normal sperm physiology, their elevated levels can lead to oxidative stress, negatively impacting sperm motility, viability, morphology, mitochondrial membrane potential (MMP), acrosomal integrity, and overall sperm functional membrane integrity. The balance between these reactive species and the antioxidant defense mechanisms is crucial for optimal reproductive performance. Sodium nitroprusside (SNP), as a NO donor, has demonstrated varying effects on sperm function depending on its concentration, highlighting the complexity of NO's role in reproduction. At low concentrations, NO can enhance sperm motility and viability, but at higher levels, it becomes detrimental, leading to decreased motility, impaired acrosomal reaction, and damage to

MMP. The interplay between ROS and NO suggests that both oxidative stress and nitrosative stress must be carefully regulated to prevent damage to sperm cells and ensure successful fertilization. Future research should focus on developing strategies to modulate ROS and NO levels in semen to improve artificial insemination outcomes in buffaloes. Understanding the precise mechanisms through which these reactive species affect sperm functions will be key in enhancing fertility and reproductive efficiency in this valuable livestock species.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that there was No generative AI technologies used and No text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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