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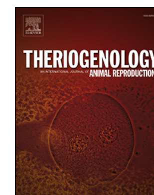

# Bimodal interplay of reactive oxygen and nitrogen species in physiology and pathophysiology of bovine sperm function

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ABSTRACT

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the mediators of redox activity and are known to perform concentration-specific bimodal roles. At lower concentrations, serves as a molecular messenger and signaling molecule while at higher concentrations induces stress which in turn alters the sperm's functional characteristics. Production of ROS and RNS cannot be prevented entirely and should not be followed as a pragmatic approach as they are involved in numerous sperm physiological functions. When the antioxidants defense armory is meager, excess generation of these species cross the physiological limits and inactivates essential metabolic enzymes and disrupts signal transduction altering normal sperm functions. As per the available literature, oxidants mostly arise as a result of pathological conditions or cryopreservation-induced injury. Dead and debilitated or abnormal spermatozoa and associated leukocytes release free radicals in an excess amount which elicits oxidative and nitrosative stressors that are potentially toxic to cryosurviving sperm. ROS plays a double edge sword effect on sperm function, as regulators of physiological mechanisms at low levels and as toxicants when produced at high concentrations. Recently nitric oxide (NO·) has emerged as a potential regulator of sperm physiology, in addition, found to mediate homeostasis of the seminal plasma microenvironment when semen samples are incubated with optimal concentrations of NO· compounds. The NO· compounds can provide some resistance to future stresses which are not usually harnessed by using the defensive strategy of supplementing antioxidants. Therefore, through the optimized addition of NO· donor and inhibitor in extender, the free radical-induced damage can be avoided without inhibiting their essential physiological effects on fertilization and subsequent embryo development. This article is intended to describe the role of reactive oxidants in the physiology and pathophysiology of spermatozoa and their relationship with various seminal attributes.

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## 1. Introduction

Semen cryopreservation led to a considerable reduction in geographical barriers to breed animals by the transportation of viable sperm over long distances [1]. In addition, cryopreservation has allowed for rapid genetic improvement of important farm animals as well as the control of venereal diseases which have a huge impact on the sustainability of the agri-food industry. Artificial Insemination (AI) with cryopreserved semen allows rapid genetic improvement of future progeny in a non-descript cattle population [2]. However, the decreased fertilizing ability of cryopreserved spermatozoa is a critical constraint in the intensive dairy production system. For AI, successful prolongation of spermatozoa's productive life can only be accomplished through effective cryopreservation techniques and the development of semen diluents that sustain sperm function post-thawing. Semen cryopreservation alters the sperm physiology by disrupting the physical characteristics, altering the metabolic pathways, enzymes and antioxidant status that compromises fertilizing efficiency and decreases the success rate of AI [3]. The stress associated with

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cryopreservation and thawing can be decreased either through proper understanding of molecular derangements or standardizing the cryopreservation protocol to reduce cryo-damage [3–5]. Moreover, as a result of processing and cryopreservation, dead and morbid spermatozoa release free radicals that are potentially toxic to the cryosurviving sperm cells and responsible for inflicting oxidative and associated nitrosative stress on sperm cells [6,7]. Spermatozoa are extremely prone to free radical-induced damage as they contain a higher concentration of polyunsaturated fatty acid (PUFA), modest antioxidant defenses, high content of oxidizable substrates [8] and lack antioxidant protection along with appropriate armory of defensive enzymes during the storage period [9]. The imbalance between the amount of ROS or RNS produced and detoxified decides the seminal oxidative stress and consequential molecular damage; therefore, it is a potential mediator of physiological sperm functions [10]. A recent study by Naskar [11] and Mohammed et al. [12] determined that *in vitro* addition of RNS compounds at optimal concentrations both in fresh as well as cryopreserved semen promotes sperm functional integrity and lowers the deteriorations in frozen-thaw semen. Physiological concentrations of ROS or RNS have positive effects on sperm function. This review focuses on the role of ROS and RNS in the physiology and pathophysiology of bovine spermatozoa with its potential effects on semen quality.

## 2. Cryopreservation and associated stressors

Cryopreservation involves the combination of controlled temperature reduction and cellular dehydration. Semen cryopreservation induces extensive biophysical and biochemical alterations resulting in destabilization and epigenetic alterations in the structures of the spermatozoa [13]. In addition, cryopreservation induces changes in the metabolome profile of bovine semen both at plasmatic and cellular compartments [14]. The stressors primarily involved are, sudden osmotic and temperature changes [15], deleterious effects of media components and cryoprotectants, generation of free radicals and depletion of antioxidants [16]. Elevated intracellular calcium in cryopreserved viable human and bull sperm reflects impaired mitochondrial membrane fluidity and subsequently increases mitochondrial membrane potential to induce ROS release [17]; therefore, cryopreservation serves to be a common root cause of oxidative stress. In addition, higher sperm intracellular calcium levels activate a signaling mechanism that is associated with cryocapacitation [18]. During cryopreservation, the loss of membrane integrity of apoptotic and dead spermatozoa further elicits the generation of ROS particularly in egg yolk-based extenders affecting the viable sperm cells in the immediate surrounding [19]. Furthermore, the freeze-thaw cycle induces nuclear damage and genome alteration causing irreversible degradation of the sperm during the process of cryopreservation [20,21]. Several studies support the fact that there are no functional DNA repair mechanisms [15,22], and enzymes [23] during cryopreservation and frozen-thawed sperm are more vulnerable to free radicals attack as compared to other cells [24].

## 3. Peroxiredoxins (PRDXs) and antioxidants additives in semen diluents

Antioxidants manifest important mechanisms *in vitro* and *in vivo* by breaking the oxidative chain reaction or by directly quenching the free radicals, facilitating the maintenance of cellular homeostasis. During epididymal transit, sperm undergo morphological and biochemical transformations, as a result, cytosolic antioxidant enzymes remain in low amounts in the maturing spermatozoa providing minimal antioxidant protection [25]. Since the presence of antioxidants is important to circumvent oxidative damage in the spermatozoa, the limited antioxidant enzymes and peroxiredoxins (PRDXs) family appear to be a key participant in antioxidant defense [26,27].

PRDXs are antioxidant enzymes and ROS modulators that have been shown to play a key function in redox signaling and preventing oxidative damage in human [25] and bull [28] spermatozoa. The six members of the PRDXs family are differentially localized in subcellular compartments of the human spermatozoon (head, mitochondrial sheath, and flagellum), with PRDX6 being the most abundant and present in all compartments [25,29]. PRDXs are SH-dependent, selenium and heme-free peroxidases with one or two cysteine residues at the active site and are highly expressed in all living species [30,31]. These acidic proteins can reduce both organic and inorganic hydroperoxides, and peroxynitrite by coupling with the thioredoxins reductase system [29,30,32]. PRDX6 is a bifunctional enzyme with peroxidase and calcium-independent phospholipase $A_2$ ($iPLA_2$) activities which are necessary to remove noxious free radicals and repair oxidized membranes [33]. It is the primary antioxidant defense in human spermatozoa, maintaining viability, mitochondrial function and DNA integrity [29,34]. It plays a role in the regulation of sperm activation and ensures early capacitation events that are important for fertility in humans and mice [35,36]. PRDX6 prevents the spermatozoa from entering the truncated apoptotic cascade pathway by regulating ROS generation and activating the phosphoinositide 3-kinase (PI3K) and its target AKT (protein kinase B) pathway [27]. Unsaturated fatty acids, such as arachidonic acid (AA) and lysophospholipids such as lysophosphatidic acid (LPA) are produced by PRDX6 calcium-independent phospholipase $A_2$ (PRDX6 $Ca^{2+}$-$iPLA_2$), which activate the PI3K/AKT pathway and inhibit apoptotic-like alterations that lead to spermatozoa death [29]. Strong oxidative stress promotes peroxidase inactivation by interacting with the thiols in PRDXs active sites, causing their oxidation and consequent enzyme deactivation [37]. Low levels or inactivation of PRDX1 and PRDX6 due to thiol oxidation are linked to aberrant sperm parameters *viz.* poor motility, lipid peroxidation, DNA damage, and infertility [34]. It is also reported that the absence of PRDX6 in spermatozoa compromises semen quality both *in vivo* and *in vitro* [38,39] and increases the levels of post-translational protein modifications (S-glutathionylation and carbonylation) and damages the sperm chromatin [38]. Although scanty information is available about its role in bovine sperm functions, its presence in the seminal plasma and positive relation with % viability demarcate it as a potential marker of sperm cryotolerance [40]. Thus to minimize the cryopreservation-induced injury and to optimize the levels of reactive species, optimum combination and concentration of antioxidants are essential along with adequate freezing-thawing protocols.

The exogenous addition of different enzymes and antioxidants has been investigated to neutralize oxidants [41] and to maintain the motility of cryopreserved bovine sperm [42]. Beconi et al. [43] in their pioneering research on bovine semen reported that the inclusion of non-enzymatic natural antioxidants (vitamin E @ 1 mg/mL and ascorbic acid @ 5 mM) in the diluent exerted a protective effect against lipid peroxidation during freezing and thawing, thereby preserving the metabolic activity and cellular viability. Latter O'Flaherty et al. [44] and Dalvit et al. [45] in their study observed that there was a significant reduction in the % of capacitated spermatozoa and fertilization rate while no change in progressive motility and the % of live spermatozoa when incubation medium contained vitamin E and ascorbic acid. They concluded that increasing vitamin E concentration and preserving its functionality with ascorbic acid could affect the physiological production of reactive oxygen species, specifically superoxide anion, affecting the fluidity and permeability of the plasma membrane. Therefore, altering capacitation, acrosomal reaction, and *in vitro*

fertilization processes. Hezavehei et al. [46] recently reviewed that supplementing the freezing media with various antioxidants (enzymatic, non-enzymatic, herbal, synthetic, cholesterol-loaded cyclodextrins, etc.) improves sperm motility, viability, DNA integrity, acrosomal reaction, membrane integrity and decreases lipid peroxidation and ROS production. Since the oxidative stress associated with freezing and thawing primarily manifests mitochondrial dysfunction, various non-enzymatic antioxidants and bioactive agents targeting mitochondria like mito-tempol [47,48] mitoquinone [49], quercetin [50], resveratrol [51], melatonin [52,53], elamipretide [54,55], etc. have been reportedly found to alleviate the peroxidative damage. In particular, mito-tempol acts as an intracellular scavenger of ROS (superoxide anions) even in the presence of an oxidative inducer and is found to be the only antioxidant agent effective in nullifying $H_2O_2$ [56]. Correspondingly melatonin a time and dose-dependent antioxidant alter mitochondrial respiratory complexes I and IV, efficiently scavenging hydroxyl and peroxyl radicals and increasing sperm motility [57,58].

## 4. Reactive oxygen species (ROS)

ROS are the by-products of the normal metabolism of aerobic organisms which includes superoxide anion ($\cdot O_2^-$) as a primary molecule that transforms into various secondary products like singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), peroxyl radical ($ROO^-$) and hydroxyl radicals ($OH^-$) [59,60]. They are either free radicals or non-radical molecules, additionally derived from the reaction of carbon-centered molecules with ROS in all complex cellular systems [60]. The dead spermatozoa, abnormal spermatozoa, leukocytes, immature sperm cells in semen [6] and mitochondria in spermatozoa are the endogenous sources and inflammatory reactions or diseases of the male genital tract are the exogenous sources of ROS production [61]. Intracellular ROS is produced through enzymes such as NADPH oxidases in the cell membrane and cytochrome P450-dependent oxygenases in mitochondria and endoplasmic reticulum, or by direct electron transfer to oxygen in the electron transport chain in mitochondria [62]. Recent studies have reported that energy metabolism (oxidative phosphorylation pathway) and oxidative deamination of the aromatic amino acids of egg yolk by the enzyme L-amino acid oxidase of spermatozoa can also be the source of ROS [60]. The mitochondrial ROS production is bidirectionally driven by oxidative stress which triggers lipid peroxidation cascades that terminate in the generation of small-molecular-mass aldehydes which covalently bind proteins at vulnerable histidine, lysine, and cysteine residues and ends up activating a self-perpetuating chain of free radical formation [63]. Sperm-specific ROS mainly originates from the mitochondrial electron transport system, which enhances during abnormal oxygen supply and cryopreservation-induced mitochondrial dysfunction [17,64]. Likewise, an aromatic amino acid oxidase-catalyzed reaction is the principal source of ROS production in bovine semen containing dead and abnormal spermatozoa [65], and leukocytes like neutrophils and macrophages are associated with excessive production of ROS [66,67]. The impulsive generation of intracellular ROS from dead sperms affects the cryosurviving sperm, which can be observed in routine semen analysis like seminal plasma hyperviscosity [68] and poor sperm membrane integrity [61]. Seminal plasma confers some degree of protection against ROS damage due to the presence of catalase and superoxide dismutase enzymes that scavenge ROS. The absence of endogenous defence mechanism and exposure of gametes to various manipulation techniques as well as environment contribute to the accumulation of ROS during *in vitro* conditions [69]. In addition, adverse climatic conditions like heat stress [70] and dissolved oxygen in the extender enhance the production of ROS [5]. ROS is detrimental from a reproduction perspective, as they generate furthermore free radicals, thereby perpetuating a chain of reactions and creating high amounts of oxidants around viable sperm pools [71].

ROS plays a double edge sword effect on sperm function, as regulators of physiological mechanisms at low levels and as toxicants when produced at high concentrations (Fig. 1.). The controlled endogenous generation of ROS by germ cells undoubtedly plays a vital role in the regulation of sperm count and quality during the spermatogenesis and post-testicular maturation phase [67,72]. Low levels of ROS *viz.* “oxidative eustress” play crucial roles in signaling processes and normal sperm physiology [6,73]. In contrast, excess ROS generated during cryopreservation attack viable sperm PUFA rich membranes, impair DNA, alter seminal antioxidant profile, and induce capacitation-like changes resulting in the low fertilizing ability of sperm [16,63].

### 4.1. Physiological roles of ROS

Low or moderate ROS levels in the body maintain a functional redox state and serve important functions such as maintenance of primary cellular antioxidants, physiological regulation of cell signaling, cellular growth, regulation of cytokines, neuromodulation, immune modulation and regulation of gene expression [41,74]. ROS through redox modulation of cysteine residues influence the function of ion channels and transporters, modulate kinases and activate the ubiquitin-proteasome system, which is important in many redox-regulated activities and cell signaling [19,41]. The physiological role of these molecules is crucial in reproductive events such as sperm maturation, hyperactivation, acrosome reaction and sperm-oocyte fusion [75,76] with their short half-life and limited diffusion. ROS generate cyclic AMP in spermatozoa and optimize tyrosine phosphorylation cascades in the female genital tract, resulting in hyperactivation [9] and binding to the zona pellucida [77].

ROS promote tyrosine phosphorylation mediated capacitation via increasing cholesterol efflux, bicarbonate inflow, calcium influx, and other small ion influx, which causes hyperpolarization, activates adenyl cyclase, inhibits phosphotyrosine phosphatases, and activates tyrosine kinases [76]. Capacitation is part of an oxidative process and the superoxide anion promotes physiological capacitation in cryopreserved human [78] and cattle [79] spermatozoa. In addition, a certain optimal concentration of ROS is necessary for increasing fluidity of the membrane and normal sperm functioning [80,81]. During spermatogenesis, ROS like $H_2O_2$ confers protection to mitochondria by inducing changes in its membrane marked by the formation of the mitochondrial capsule. Under mild oxidative conditions, disulfide bonds develop between the cysteine residues of protamine, resulting in characteristically tight packaging of chromatin and the stabilization of nuclear DNA, which may aid in sperm maturation [75,82]. Therefore at controlled physiological concentrations, ROS stabilizes the mitochondrial capsule [83], serves as an essential second messenger in the sequence of coordinated molecular events and fertilization [76,84]. Another important aspect of physiological concentrations of ROS that it leads to activation of the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) enzyme, which promotes sperm survival, maintains them in an active state and inhibits apoptosis [63,85].

### 4.2. Pathological effects of increased ROS

Oxidative stress arises due to the excess generation of ROS during freezing and thawing procedures and lack of intracellular alleviation measures is the foremost factor behind the impaired quality of frozen-thawed sperm [86]. Throughout various

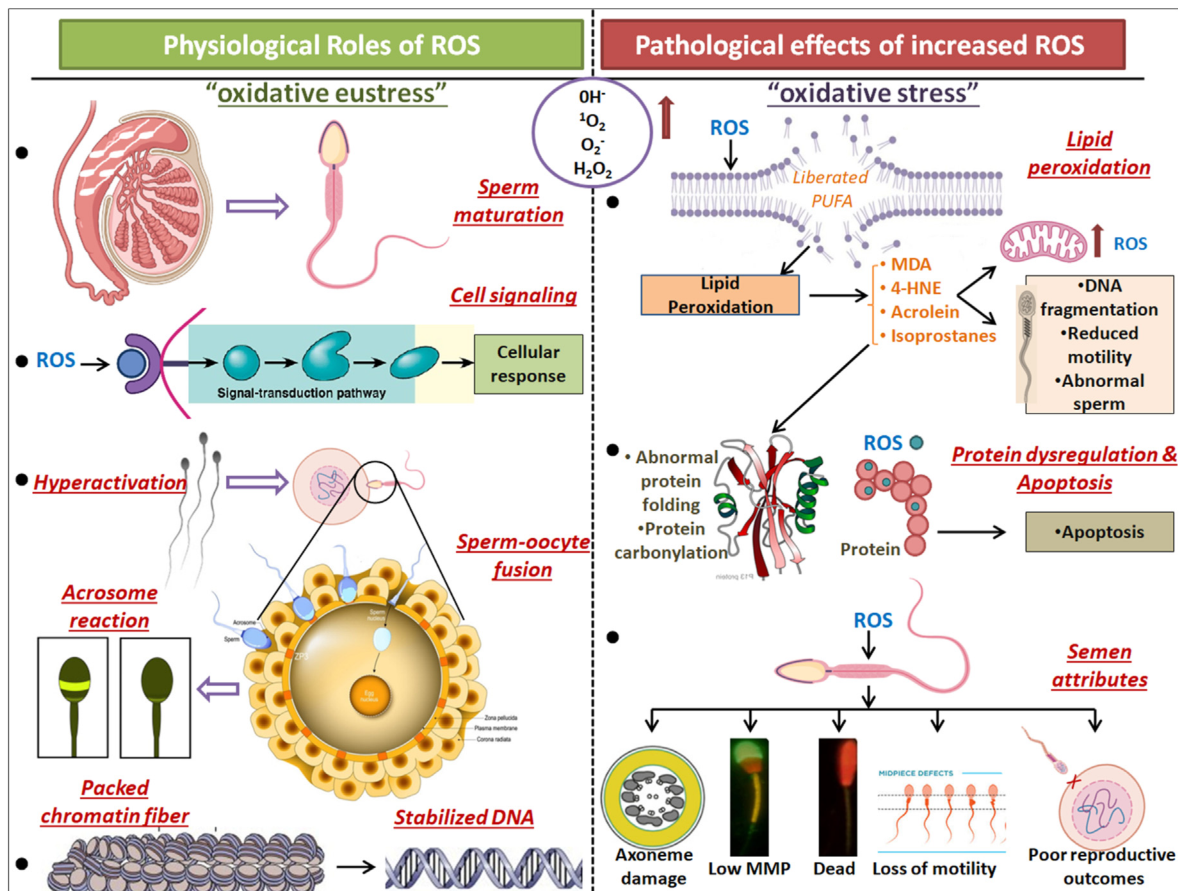


**Fig. 1.** Physiological roles of ROS and pathological effects of increased ROS on bovine sperm functions. Note: ROS, Reactive oxygen species; PUFA, Polyunsaturated fatty acid; MDA, Malondialdehyde; 4-HNE, 4-Hydroxynonenal; MMP, Mitochondria membrane potential, $H_2O_2$, Hydrogen peroxide; $OH^-$, Hydroxyl ion; $^1O_2$, Singlet oxygen; $O_2^-$, Superoxide anion(Created with BioRender.com).

operations before freezing to AI (*in vitro* manipulation), bovine sperms are exposed to light and aerobic environments, generating oxidative radicals [87]. ROS are unstable and highly reactive throughout the metabolism process, they get stable on gaining electrons from nucleic acids, lipids, proteins, carbohydrates, or any other adjacent molecule, culminating in a chain reaction leading to cell damage [63,88]. Lipid peroxidation and stimulation of mitochondrial ROS results in the consequent generation of lipid aldehydes which has a protein-adducting activity that culminates in a self-perpetuating redox cycle and a state of oxidative stress [89]. Mitochondria being the chief source of ROS and ATP production, under limited antioxidant defence, the oxidants impair the electron transport chain, resulting in decreased ATP synthesis, altered mitochondrial membrane permeability, disruption of calcium homeostasis and ultimately challenging the sperm competence [17]. Sperm may undergo an intrinsic apoptotic pathway by opening the permeability transition pore and consequently extrusion of cytochrome *c* [90]. Redox-dependent genetic and protein modifications have been associated with oxidative stress in humans, bovine, equine, hamster and mouse resulting in low sperm quality and fertility failure [37,91]. As a consequence of redox deregulation, there is irreversible oxidation of thiols in cysteine residues of key proteins involved in controlling numerous sperm functions, leading to malfunction and ultimately death of the spermatozoa [92]. In addition, the seminal antioxidant armoury is also incompetent to maintain equilibrium between the production and detoxification of ROS [93]. The intrinsic reactivity by $H_2O_2$ and the $\cdot O_2^-$ have been proposed as a major cause of defective sperm functions in case of male infertility [94]. The following are the adverse effect of ROS on sperm functions.

a. Lipid peroxidation and DNA damage

The sperm membrane is rich with PUFAs which contribute to the membrane elasticity and fluidity that is required for successful fertilization, however, at the same time, it makes sperm extremely vulnerable to peroxidative damage [95,96]. The abundance of carbon-carbon double bonds, which are easily oxidized by ROS results in the loss of more than half of the fatty acids and susceptibility to peroxidative damage [76]. Free radicals react with the fatty acid chain to form lipid radicals which further react with oxygen to form peroxyl radicals which can extract the hydrogen from the lipid molecules, causing an autocatalytic self-propagating reaction associated with destabilization of the membrane [65,76]. This lipid peroxidation cascade eventually terminates in the generation of highly reactive lipid peroxidation products such as malondialdehyde (MDA), 4-Hydroxynonenal (4-HNE), acrolein and isoprostanes which ultimately results in loss of membrane permeability and membrane potential, and also affects the cellular integrity [97]. The loss of membrane properties results in decreased fluidity, increased non-specific permeability to ions and alteration in receptor transduction, transport process and membrane enzymes [98]. The whole process of lipid peroxidation causes several downstream effects like increased DNA fragmentation, reduced

plasma membrane integrity, reduced progressive motility and increased morphological abnormality of bovine spermatozoa [99].

ROS induce epigenetic changes in spermatozoa and are associated with a dysregulation in levels of mRNAs [97]. ROS brings about DNA damage by reacting with the heterocyclic DNA bases and sugar moiety and causes modification of all bases, production of base-free sites, frame shifts, deletions, formation of DNA cross-links, chromosomal rearrangements and chromosomal micro deletions [100,101]. Specifically, free radicals bind to the double-bonds of DNA bases and extract hydrogen from deoxyribose carbon leading to strand breaks, base release and numerous base alterations which certainly have an impact on embryonic development [62]. The presence of PUFAs, particularly docosahexaenoic acid (DHA) with six double bonds per molecule, and the lack of apurinic/apyrimidinic endonuclease 1 (APE1), an enzyme that plays a critical role in DNA repair in the base excision repair pathway, are the mechanisms behind spermatozoa's susceptibility to the afore mentioned damages [102,103]. A significant positive correlation was reported between high semen oxidative stress levels, DNA damage and reduced fertilizing capacity, specifically in infertile human patients with high-frequency DNA strand breaks [80,104,105], precisely single and double-strand DNA breaks [106]. Oxidative stress-induced DNA fragmentation in the sperm nucleus, mediated by peroxidative damage not only disrupts sperm fertilizing potential but also retards the development of a healthy embryo [107]. The extent of DNA damage in mature sperm is also directly related to ROS production by immature spermatozoa, higher the ROS production lower the percentage of mature spermatozoa [108,109].

b. Protein dysfunction and apoptosis

The generations of cytotoxic aldehydes from lipid peroxidation results in additional damage to spermatozoa as these aldehydes covalently binds to exposed lysine, histidine, and cysteine residues on target proteins [63], and disrupt post-translational modification processes. Alterations in post-translational modification processes result in a disturbance in protein-protein interactions, protein localization and protein turnover which marks incorrect cleavage of proteins, changes in protein folding pattern and protein carbonylation [60]. Moreover, direct oxidation or aldehyde adduction to the protein components of the mitochondrial electron transport chain increases the cell's inclination to generate excess ROS, causing additional proteolytic degradation perturbing cell functionality [64,110]. Produced ROS can bind to proteins and either activate or deactivate leading to the induction of the intrinsic apoptotic cascade [111] which may be the sequel to the depleted ATP and inevitably leads to cell death [112]. The intrinsic apoptotic pathway may further originate signals that initiate the activation of caspases leading to the formation of adducts with mitochondrial proteins and DNA fragmentation [113]. This is often accompanied by loss of mitochondrial membrane potential, translocation of phosphatidylserine, destabilization of nucleoprotein structure and oxidative DNA damage induced alterations to gene expression [89,114].

c. Effect on seminal attributes

The motility of sperm is lost more abruptly when exposed to higher oxygen levels compared to nitrogen due to excess production of ROS [5,115] and lower detoxification [116]. Excessive production of ROS in semen can cause insufficient axonemal phosphorylation, ATP depletion, decrease sperm viability and midpiece sperm morphological defect affecting motility [117]. In addition, induce premature acrosome reaction, lipid peroxidation and inhibit mitochondrial membrane potential leading to low fertilization rates [6,24,118]. The physiological concentrations of ROS are disrupted during *in vitro* manipulation in assisted reproductive techniques (ARTs), as dead and damaged spermatozoa itself generates supra-physiological levels of ROS that are detrimental to viable sperms [69]. As ROS levels are highly connected with the conception rates in humans undergoing IVF, measuring seminal ROS levels has become important [119]. Higher levels of ROS can be considered as a potent marker of male infertility, independent of normal or abnormal seminal parameters [120]. The status of seminal oxidants level is emerging as a significant diagnostic and prognostic tool crucial for the treatment and management of oxidative stress. High ROS levels are due to the low anti-oxidative capacity of sperm as most of their cytoplasm-containing antioxidants are discarded during the terminal stages of spermatogenesis or diluted during the extension of semen for producing a large number of straws for AI [121,122]. Therefore, accurate measurement of concentrations of reactive species and addition of an antioxidant to the extender can be an appropriate approach to control ROS-induced oxidative stress [58,123]. In addition to *in vitro* sperm processing and storage; heat stress, nutrition and age are emerging as potential causes of oxidative stress in mammalian spermatozoa [60]. Thus a molecular understanding of semen redox biochemistry and the use of this knowledge to devise processing and storage protocols with optimum antioxidant supplement strategies is a prerequisite for efficient ROS homeostasis.

## 5. Reactive nitrogen species

Reactive nitrogen species (RNS) is a subset of the ROS family which include nitrogen dioxide ($NO_2$), peroxynitrite ($ONOO^-$), nitric oxide ($NO^\cdot$) and all those products that are formed by the interaction of $NO^\cdot$ to $^\cdot O_2^-$ and $RO^\cdot$ [124]. RNS is particularly prevalent in several parts of the male reproductive system and their origin can be classified based on their structure and cell types. They are ubiquitously present throughout the male reproductive system *viz.* Testes, epididymis, accessory glands, penis (infiltrating leukocytes, epithelial cells, endothelial cells, smooth muscle cells and macrophages) and ducts (ejaculatory duct and vas deferens) [71]. The mitochondria are the prime site for the formation and reactions related to nitric oxide and peroxynitrite, the formation of the latter precedes at a cellular level when the level of nitric oxide overwhelms superoxide dismutase [125]. Peroxynitrite further reacts with other molecules and forms additional reactive nitrogen species such as nitrogen dioxide ($NO_2$) and dinitrogen trioxide ($N_2O_3$) as well as other chemically reactive nitrogen free radicals. $NO^\cdot$ being a diffusible free radical, functions as an intracellular messenger in a variety of physiological and pathological conditions [126]. RNS plays a physiological role in signal transduction by inducing mitogen-activated protein (MAP) kinase signaling pathways [71], immune system facilitation [127,128], and mediating tight junction dynamics of the blood-testis barrier, which is important for spermatogenesis, germ cell maturation, and development [127,129,130]. Although it supports several sperm functions at physiological levels [131,132], RNS at disproportionate levels contributes to nitrosative stress, exerting severe pathological effects on the male reproductive system [133,134]. In particular, RNS has recently been implicated in inducing poor sperm function and fertilizing ability when present at the supra-physiological concentration [135] because of their ability to modify key biomolecular systems through oxidation, nitrosylation, and nitration [124]. Excessive generation of reactive oxidants is implicated in the etiology of male infertility, and even antioxidant therapy has failed to treat male infertility in many cases [135]. This led to the discovery that ROS and RNS are required for appropriate sperm functioning in small and controlled physiological concentrations.

### 5.1. Nitric oxide (NO·)

NO· is a diatomic free radical that, due to its non-polarity, may easily diffuse across phospholipid bilayer, acts as a cell-signaling molecule in mammalian cells and influences key processes in vital body systems [136–138]. Owing to its potent vasodilation property, NO· is recognized as a vascular endothelium-derived relaxing factor. Normally produced during nonadrenergic-noncholinergic neurotransmission, therefore, plays an important role in erection in males [139]. Currently, it is one of the widely investigated radical species in medicine and has significant implications in biochemical systems due to its versatility and extensive downstream network [113]. NO· biosynthesis has been reported in several organs of the male reproductive system [140,141] including spermatozoa [142,143]. The NO· is produced in sperm from L-arginine via the enzyme nitric oxide synthase (NOS) and the activity of the enzyme depends on male germ cell maturity [144]. All identified mammalian NOS isoforms are heme-containing proteins with a monomeric molecular mass of 126–160 kDa in natural conditions. NOS comprise a reductase domain-containing tetrahydrobiopterin (BH4), which through protein-protein interactions catalyze the reaction that produces NO· [135]. Apart from NOS, the said reaction needs oxygen, as well as cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calmodulin and calcium to produce NO· and L-citrulline as a byproduct [128,145]. The three different isoforms *viz.* endothelium NOS (eNOS) is mostly found in the endothelium, neuronal NOS (nNOS) is predominantly found in neural tissue and inducible NOS (iNOS), found in macrophages, neutrophils and hepatocytes [136] which was also reported to be expressed in the head or flagellum regions of the sperm [146,147]. Therefore, the endogenous presence of eNOS, iNOS, and Nnos within the testis proves the importance of NOS for spermatogenesis. The isoforms eNOS and nNOS are constitutive and biosynthesized at steady rates are recognized as calcium ($Ca^{2+}$) calmodulin-dependent enzymes [131,136]. Whereas, iNOS is a $Ca^{2+}$independent enzyme that has specific inducers (e.g. inflammatory cytokines, tumor necrosis factor and bacterial endotoxins) that vary from cell to cell. The above factors elevate iNOS synthesis and activate for a longer duration, which can result in the production of higher concentrations of NO· [148]. TnNOS, a testis-specific subtype of nNOS, has been identified as a substantial contributor to the synthesis of NO· [129,130], localized in leydig cells and engaged in steroidogenesis [149]. Moreover, some research has postulated the indirect role of glucose through the pentose phosphate pathway and arginase enzyme (mitochondrial form) in the regulation of the synthesis of nitric oxide.

NO· has been demonstrated to have effects via two separate signaling pathways: cyclic guanosine monophosphate (cGMP)-dependent (classical signaling) and cGMP-independent (non-classical signaling) [148,150]. Classical signaling involves activation of soluble guanylate cyclase (sGC), generation of cGMP and subsequent activation of specific cGMP-dependent enzymes, while non-classical signaling occurs through covalent post-translational modification of target proteins, that is S-nitrosylation, S-glutathionylation and tyrosine nitration [151–153]. NO· regulates its activity via a feedback inhibition mechanism [127] which is highly dependent on its concentration within the male reproductive system. While abnormal concentrations of NO· undoubtedly affects reproductive efficiency, it is least intoxicating with a short half-life of 3–10 s, and quickly combines with oxygen and transforms to nitrite and nitrate in tissues. Therefore, it has a paradoxical bimodal role (Fig. 2.) in the physiology of bovine spermatozoa depending on time and concentration, resulting in either beneficial or detrimental effects on the sperm pool.

#### 5.1.1. Physiological roles of NO

NO· is the regulatory molecule in controlling the hypothalamic-pituitary-gonadal axis [137]. It stimulates the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which then potentiates the pituitary gland to secrete the gonadotropins by triggering the pituitary localized nNOS [154]. NO· also acts as a major facilitator of human sexual behavior; as it causes penile erection in males by producing cGMP in the corpora cavernosa [155], relaxes the vaginal smooth muscle and secretes vaginal discharges in females [154]. Owing to its potent junction modulation property and ability to control the level of cytokines and hormones in the testis, it has a typical role in modulating germ cell viability and development [113]. At physiological levels, NO· has an essential role in sperm transport from the rete testis to the epididymis and in regulating motility, viability, morphology and the ability of sperm to capacitate and undergo acrosome reaction [130,135]. NO· accomplishes beneficial effects on sperm function either by improving the functional status of sperm mitochondria and sperm quality [156] or by scavenging, deactivating and inhibiting free radicals [157]. The concentration <1 μM increases normal sperm motility and has shown sperm hyperactivation [132,156]. In consonance with previous findings, improved motility, viability, membrane integrity, acrosome integrity and reduced total abnormality were reported after *in vitro* addition of an optimized dose of NO· compounds post thawing [11,158]. Khodaei et al. [159] and Upadhyay et al. [160] recorded that *in vitro* addition of nitric oxide compounds at standardized concentrations in cryopreservation media significantly improves various seminal attributes of bovines after freezing.

#### 5.1.2. Effect of nitric oxide compounds on seminal attributes

RNS have major contribution to human reproduction since its recognition as a key molecule involved in the *in vivo* regulation of the hypothalamic-pituitary-gonadal axis and *in vitro* regulation of sperm functions when added at an optimized concentration (Table 1.). Nitric oxide due to its dual action in humans, impairs sperm morphology at high concentration, while at low concentration (1–500 μM) enhances sperm motility [161]. The amount of nitric oxide produced by sperm cells and how long it lasts determines whether it has a pathological or physiological effect. The ability of NO· to interact with the Iron-Sulphur (Fe–S) center of aconitase and other mitochondrial electron transport chain enzymes such as NADH dehydrogenase or succinate oxidoreductase decreases motility and viability of human spermatozoa particularly at high concentrations [162] by altering energy, metabolism, and cellular respiration [163]. Herrero et al. [131] reported that capacitation was accelerated by NO· releasing compounds due to an increase in the levels of tyrosine phosphorylation of two different sperm proteins (p81 and p105). In cryopreserved bovine spermatozoa, exogenous NO· functions as a capacitation inducer and participates in intracellular pathways that lead to the activation of protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinase (PTK) [164]. The precursor of NO· synthesis, L-arginine, affects polyamines which are key biomolecules for cell growth and differentiation [165], increasing sperm motility [166], vigor, membrane integrity and oocyte penetration [167]. The sodium nitroprusside (SNP) and N-nitro-L-arginine methyl ester (L-NAME) are L-arginine analogues that function as NO· donor and inhibitor, respectively. Among different NO· donors, SNP is regarded as the most effective for *in vitro* study of the action of NO· [168]. The SNP treated semen samples (up to 100 nM) in human [169] and Holstein bull [159] maintain motility, viability and acrosome integrity of spermatozoa at pre and post-thawing stages. Contrarily some researchers observed that the *in-vitro* SNP addition increases ROS production, induces precocious acrosome reaction and significantly

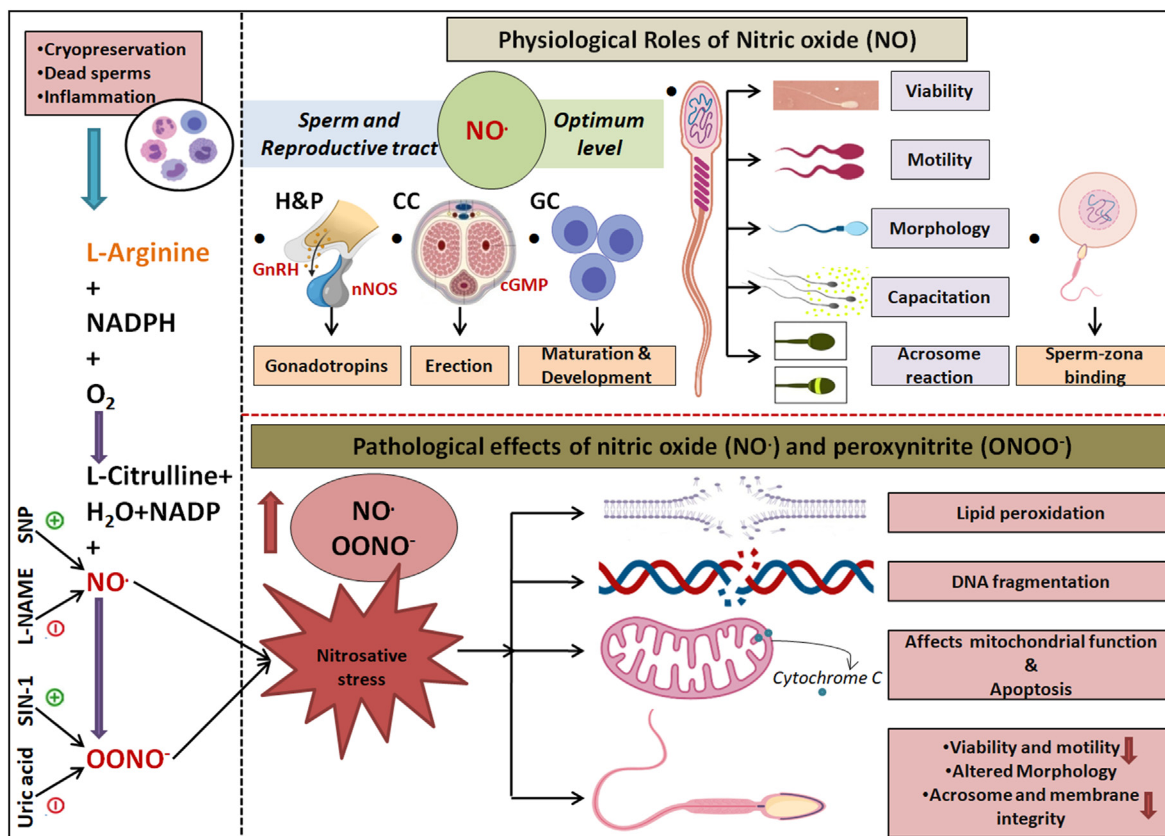


**Fig. 2.** Physiological roles of nitric oxide, sources of reactive nitrogen species and pathological effects of increased nitric oxide and peroxynitrite on bovine sperm functions. Note: H&P, Hypothalamus and pituitary; CC, Corpus cavernosum; GC, Germ cell; NADPH, Nicotinamide adenine dinucleotide phosphate; NADP, Nicotinamide adenine dinucleotide phosphate, SNP, Sodium nitroprusside; L-NAME, N-nitro-L-arginine methyl ester; SIN-1, 3- Morpholinosydnonimine; nNOS, neuronal Nitric oxide synthase; GnRH, Gonadotropin-releasing hormone; cGMP, cyclic Guanosine monophosphate(Created with BioRender.com).

decreases the rates of fertilization with compromised embryonic development [170]. Digamber et al. [171] investigated the influence of SNP treatment on frozen-thawed buffalo semen and observed fewer numbers of normal spermatozoa with reduced viability and motility as compared to control. High concentrations of NO· damage the nuclear or mitochondrial DNA of spermatozoa via deamination, nitration and oxidation process [71].

Rodriguez et al. [164] reported that certain NOS inhibitors have proven essential for assessing the role of NO· in physiological processes. The L-arginine analogues such as N-nitro-L-arginine (L-NA) or L-NAME are specific inhibitors of constituent and inducible nitric oxide synthase (NOS) forms as they compete with L-arginine for the limited number of binding sites on the enzyme eNOS, whereas aminoguanidine (AG) selectively inhibits inducible NOS [180]. In cattle and buffalo, the addition of L-NAME to sperm capacitation media reportedly decreases sperm membrane integrity [167], inhibits premature capacitation and induces acrosome reaction 6 h after capacitation [174]. A similar study in cattle by Rodriguez et al. [164] reported that the capacitation was inhibited by the addition of L-NAME at different concentrations (0.001, 0.01 and 0.1 mM). The addition of a higher concentration of L-NAME (10 mM) has significantly ($P < 0.05$) inhibited NO· synthesis, sperm progressive motility, sperm vigor and sperm membrane integrity suggesting the importance of NO· in functional seminal attributes [167]. Interestingly L-NAME was also reported to potentiate sperm count and morphology [181], indicating its physiological role against peroxidative damage which arises during nitrosative stress. This conjectures the existence of dose and time-dependent variation in sperm motility, viability, membrane integrity, acrosome integrity and total abnormality as evidenced by various researchers in bovine spermatozoa after *in vitro* addition of SNP and L-NAME [11,158].

### 5.1.3. Pathological effects of increased NO

At supraphysiological concentration NO· produces noxious peroxynitrite and nitrosothiols by reacting with $\cdot O_2^-$ and thiol sites respectively. Another potentially crucial factor that may have NO· implications in male infertility is NOS uncoupling [136], which results in the generation of $\cdot O_2^-$ rather than NO· and a decrease in NO· bioavailability. Excessive level of NO· or dysregulation of NOS in testis triggers uncontrolled germ cell apoptosis [130]. Pathological levels of NO·, greater than 1 μM, have been involved in lipid peroxidation because of the easy abstraction of hydrogen from PUFA rich sperm plasma membranes [11,158]. It triggers a cascade of reactions, resulting in the production of free radicals that can be further oxidized to form additional free radicals. Significant levels of seminal NO· (μM) are associated with alterations in sperm functions such as decreased motility [132], inhibition of cellular respiration [162] and DNA damage [182] while a further higher concentration of NO· (mM) have been demonstrated to cause mitochondrial hyperpolarization, cytochrome *c* release and sperm cell death [183]. Some researchers reported a negative correlation between high NO· concentrations and sperm morphology [184,185].

## 5.2. Peroxynitrite ($ONOO^-$) as an oxidant

A detrimental effect of NO· is mediated by the formation of peroxynitrite [126]. $ONOO^-$ is a strong oxidant, nucleophile and

**Table 1**
Physiological and pathological roles of different concentration of nitric oxide and peroxynitrite compounds on sperm functions.

| Free Radical | Study | Result | Species | Reference |
|---|---|---|---|---|
| NO· | Incubation with SNP @ $10^{-4}$ to $10^{-6}$ M concentration over 20 h | Significant reduction in motility in a dose-dependent manner while no significant changes in sperm viability | Human | Tomlinson et al. [172] |
| NO· | Incubation with GSNO and PTIO @ 100 μM over 20 min at 37 °C | No significant change in progressive motility | Human | Miraglia et al. [173] |
| NO· | a. Addition of 10 mM L-Arg to the capacitating medium (30−60 min at 38.5 °C with a humidified atmosphere of 95% of air and 5% $CO_2$ for stabilization).<br>b. Addition of 10 mM L-NAME to the capacitating medium. | a. Increase in progressive motility, sperm vigor, sperm membrane integrity and oocytes penetration.<br>b. Decrease in progressive motility, sperm vigor, sperm membrane integrity and oocytes penetration | Cattle | Leal et al. [167] |
| NO· | a. Incubation with L-Arg in different concentrations (2, 5, 10, 15 and 20 mM) for more than 5 h (38.5 °C in 5% $CO_2$ : 95% air)<br>b. Incubation with L-NAME at the rate of 0.5 mM for more than 5 h (38.5 °C in 5% $CO_2$ : 95% air) | a. Induced capacitation and tyrosine phosphorylation of p38 (5 and 10 mM); viability and progressive motility were significantly reduced at >10 mM concentration<br>b. Heparin and L-Arg induced capacitation was inhibited significantly by L-NAME | Buffalo | Roy and Atreja [174] |
| NO· | Sperm exposed to 1 μM NO· before freezing | Significant increase in sperm kinematics and viability post thawing | Cattle | Sharafi et al. [175] |
| NO· | Addition of SNP @ 50−100 nM in diluent and examined at 1, 2 and 3 h after thawing | Increase in motility, viability, membrane functionality and acrosome integrity | Cattle | Khodaei et al. [159] |
| $ONOO^-$ | a. Incubation with different concentrations (1−20 μM) of SIN-1 for 45 min at 38 °C<br>b. Incubation with different concentrations (2, 5 and 10 mM) of uric acid for 45 min<br>c. Incubation with ≥160 mM of SIN-1 for 45 min at 37 °C | a. 10 μM concentration significantly induced capacitation with no effect on motility and viability<br>b. 10 mM concentration entirely blocked SIN-1 induced capacitation and significantly diminished heparin or SNP induced capacitation<br>c. Dose dependent decrease in motility | Cattle | Rodriguez and Beconi [176] |
| ONOO− | Incubations with SIN-1 at different concentrations (0.05, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) for up to 4 h at 37 °C | Decrease in progressive motility, total motility, mitochondrial membrane potential (significantly at 1.0 mM) with no change in viability | Human | Uribe et al. [177] |
| ONOO− | a. Incubation with 160 and 200 μM of SIN-1 for 60 min or above at 37 °C<br>b. Incubation with 10 mM of uric acid for 60 min or above at 37 °C | a. Decrease in motility, viability, membrane integrity and acrosome integrity<br>b. Increase in viability and ATP production | Cattle | Jalmeria [178] |
| ONOO− | a. Exposure of sperms to different concentrations of SIN-1 (10, 50 and 100 nM) at refrigerated temperature for 72 h<br>b. Exposure of sperms to different concentrations of uric acid (0.1, 1 and 10 μM) at refrigerated temperature for 72 h | a. Increase in metabolic enzyme activity and, dose and time-dependent decline in seminal attributes<br>b. Decrease in metabolic enzyme activity and, dose and time-dependent increase in seminal attributes | Buffalo | Kshetrimayum [179] |
| NO· | Supplementation of SNP in extender @ 1 μM and L-NAME @ 10 μM in refrigeration and cryopreserved samples | Improved motility at 6, 24 and 48 h of refrigeration and after freeze-thawing | Buffalo | Upadhyay et al. [160] |

Note: SNP, Sodium nitroprusside (NO· donor); GSNO, S-nitrosoglutathione (NO· donor); PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (NO· scavenger); L-Arg, L-Arginine (NO·-synthesis precursor); L-NAME, N-nitro-L-arginine methyl ester (NO•/NOS inhibitor); SIN-1, 3- Morpholinosydnonimine ($ONOO^-$ donor); Uric acid ($ONOO^-$ inhibitor).

highly reactive short-lived peroxide having a pka value of 6.8 and is produced from a diffusion-controlled reaction of NO· with $\cdot O_2^-$ [125,145]. Mitochondria act as a central source of $\cdot O_2^-$ (produced through aerobic respiration) in the cell and nitric oxide enters the mitochondria by diffusion from the cytosol. This favours the formation of $ONOO^-$ by an irreversible reaction because of its exothermic nature. Therefore, mitochondria act as a cellular sink of nitric oxide and the prime source of $ONOO^-$ in the cell [186]. It has been reported that much of the cytotoxicity of NO· and $\cdot O_2^-$ radical is mainly due to $ONOO^-$, as it affects mitochondrial function and triggers the pathogenic mechanism and cell death via nitration and oxidation reactions of lipids, proteins and DNA [186]. Additionally it inhibits complexes I and III of the respiratory chain [187] of mitochondria, thereby reduces the activity of the organelle and potency to produce ATP. Neither $\cdot O_2^-$ nor NO· are toxic *in vivo* as $\cdot O_2^-$ is removed by superoxide dismutase (SOD) isoforms and NO· is removed by its rapid diffusion through tissues. Therefore under normal conditions, the formation of peroxynitrite is lower [188] and its adverse effect is minimized by endogenous antioxidant defense mechanisms. But under pro-inflammatory conditions and *in vitro* manipulation, excess production of $\cdot O_2^-$ and NO· leads to the generation of significant amounts of $ONOO^-$ that in turn causes perturbations in different cellular structures (deoxyribose, glutathione, cysteine, thiols, $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase activity) and the physiological system is not efficient to minimize excess accumulation [189]. The primary pathway of nitric oxide metabolism is the peroxynitrite formation which suggests that excessive activity of NOS results in excess production of peroxynitrite. This event is marked in the idiopathic asthenozoospermia patients, in which a high level of peroxynitrite along with high nitric oxide synthase activity is present [190] that inhibits ATP production by affecting the pathways involved in both glycolysis and oxidative phosphorylation [191]. Peroxynitrite is also reported to cause the nitration of protein residues forming 3-nitrotyrosine which affects motility parameters [186]. The nitration process represents a protein modification specific for $ONOO^-$ formation *in vivo* and is widely used as a marker for peroxynitrite [177]. The increase in superoxide production observed during the cooling phase of cryopreservation without any alteration in the level of $H_2O_2$ is probably due to the drain of superoxide radical for the production of peroxynitrite. Longer exposure of spermatozoa to high levels of peroxynitrite affects viability, membrane integrity and acrosome integrity by inducing peroxidative damages [178].

During pathological conditions, both NO· and $\cdot O_2^-$ are synthesized within close vicinity in supra-physiological amount yielding $ONOO^-$. It acts as an oxidant and rather than isomerizing to nitrate, it produces nitrite and a hydroxide ion that react with cellular components *viz.* proteins, lipids and nucleic acids causing oxidative modifications of cellular components [126]. Furthermore, peroxynitrite can oxidize a sulfhydryl group producing hydroxyl radical which is found to be associated with sperm membrane lipid peroxidation [192]. Persistent generation of increased levels of $ONOO^-$ cause apoptosis or necrosis and leads to breakage of DNA strands when reacts with nucleic acids.

#### 5.2.1. Effect of peroxynitrite compounds on seminal attributes

The formation of $ONOO^-$ during cryopreserved spermatozoa capacitation and its involvement in physiological capacitation [193] through the participation of PTK is also supported by the fact that uric acid (by inhibiting $ONOO^-$) affects heparin-induced capacitation [176]. The 3- Morpholinosydnonimine (SIN-1) has been commonly used as an inducer of peroxynitrite and uric acid as a strong inhibitor of peroxynitrite in several *in vitro* studies to monitor sperm functions in humans and bovines (Table 1.). In presence of oxygen, SIN-1 transforms spontaneously to nitric oxide and superoxide [194,195]. A decrease in the motility of cryopreserved bull spermatozoa was observed after incubation with SIN-1 (3- Morpholinosydnonimine) at the rate of 160 mmol/L [176]. Uric acid possesses scavenging properties for peroxynitrite. It selectively binds with peroxynitrite excluding nitric oxide and has been shown to inhibit both *in vivo* and *in vitro* peroxynitrite-dependent processes [186]. Jalmeria [178] conducted *in vitro* experiment to evaluate the pathophysiological effect of SIN-1 and uric acid on cryopreserved semen of Karan Fries bulls. The concentration of SIN-1 $\geq$ 160 μM was detrimental for spermatozoa motility, viability, membrane integrity and acrosome integrity when incubated for 60 min or above. Conversely, 10 mM concentration of uric acid significantly increased viability and ATP production at 60 min of exposure, while there was a loss of membrane integrity and acrosome integrity at the same dose. Likewise, Kshetrimayum [179] also observed dose and time-dependent decline in seminal attributes after *in vitro* addition of SIN-1 in cryopreserved semen of Murrah buffalo bulls. Additionally, a significant increase and decrease in enzyme activity (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), which plays key roles in the maintenance of metabolic activity and membrane stability was reported after incubation with SIN-1 and uric acid respectively. The other sperm attributes increased significantly with an increasing concentration of uric acid. The possible reason behind this fluctuation can be due to the scavenging activity of uric acid on RNS and particularly on peroxynitrite, but not for ROS. A low effect was observed on sperm motility and a negative effect on membrane integrity during long exposure to the uric acid [178] may be due to species and dose variation or due to activation of other pathways by addition of this compound.

## 6. Conclusion

Information on nitrosative stress in a more rational manner, as well as their relation with cryopreservation, is highly valuable for optimizing the production of quality frozen semen. Moderate ROS levels in the body maintain a functional redox state and perform important functions in the sperm cell. Recently NO· has emerged as a potential regulator which regulates diverse sperm functions and potentiates certain sperm parameters like acrosome reaction, capacitation, hyperactivation of motility and signaling processes. Therefore, protocols with optimized inclusion of oxidants and antioxidants help to reduce the concomitant stress during extension and cryopreservation of bovine semen and to prevent any further deterioration in sperm quality during storage and post-thaw. *In vitro* and *in vivo* addition of both nitric oxide donor (SNP) and inhibitor (L-NAME) and peroxynitrite inhibitor compounds at an optimized dose found to have beneficial effects. While the action of peroxynitrite deteriorates the seminal quality, the addition of its inhibitor like uric acid in cryopreservation media can reduce its limiting effect. Concerning the optimizing activity of these compounds on seminal attributes, the degree of support provided by bovine experiments is still low to moderate. Therefore, it is necessary to standardize the dose and protocols for supplementation of these novel compounds as additives in extender in bovine and, other species and evaluate the outcomes with *in vitro* fertilization and *in vivo* conception rates. Further, requires detailed investigation through “omics” techniques to establish the pathological or physiological interactions of reactive oxidants and biomolecules at the molecular level to facilitate their extensive use in the semen cryopreservation process.

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