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PERSPECTIVE

SEMEN ADDITIVES FOR IMPROVING FROZEN-THAWED BUFFALO AND CATTLE SEMEN – A REVIEW

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Abstract

This comprehensive review delves into the evolving landscape of assisted reproductive technologies (ARTs) in bovine species, particularly focusing on the pivotal roles of semen additives in the cryopreservation of buffalo and cattle semen. In developing nations, where ARTs are still emerging, these techniques significantly influence bovine reproductive strategies. In contrast, developed regions have embraced them as primary approaches for dairy buffalo and cattle breeding. Semen cryopreservation, while offering advantages like extended storage and genetic propagation, also presents challenges. These include diminished sperm quality due to reactive oxygen species (ROS) production, alterations in sperm structure, and temperature fluctuations. Further, the effect of cryopreservation differs between cattle and buffaloes, with the latter exhibiting poorer semen viability and fertility due to inherent lipid composition susceptibilities. The generation and implications of ROS, especially hydrogen peroxide, contribute significantly to sperm DNA damage and functional impairments. To counteract these challenges, research has intensified on semen additives, aiming to bolster semen quality and protect against oxidative stress-induced damage. As the field advances, the review emphasizes the need for optimized cryopreservation techniques and tailored antioxidant strategies to harness the full potential of ARTs in bovine breeding programs..

Keywords: antioxidants; buffalo semen; cryopreservation; oxidative stress; reactive oxygen species; semen additives.

INTRODUCTION

In developing nations, assisted reproductive technologies remain nascent, with artificial insemination and cryopreservation of semen prominently influencing bovine reproductive strategies (1). Consequently, in developed regions, the predominant approach for breeding dairy buffalo and cattle involves

these techniques (2). The utility of sperm cryopreservation facilitates prolonged storage, genetic propagation of superior traits across generations, and facilitates transport over extensive distances (3). Employing frozen-thawed semen permits optimal timing of insemination without necessitating the immediate presence of the breeding male. Additionally, cryopreservation is pivotal for germplasm repository management, endorsing biodiversity conservation and safeguarding endangered species (4). Nonetheless, the effective utilization of superior male germplasm via cryopreservation encounters challenges, notably the diminished quality and fertility of semen attributed to physiological, mechanical stresses, and resultant structural alterations in sperm cells. Notably, a significant fraction of spermatozoa undergo quality compromise during cryopreservation, even when adhering to rigorous protocols, culminating in the overproduction of reactive oxygen species (ROS) (1). The primary locus of vulnerability during cryopreservation is the sperm cell's plasma membrane, which is subjected to ROS-induced lipid peroxidation and cholesterol efflux, thereby disrupting its lipid composition. Cumulative stressors, including lipid peroxidation, ice crystallization, pH alterations, and osmotic imbalances, compromise sperm motility, membrane integrity, and DNA integrity, ultimately impairing fertilization potential (5). Prior investigations have explored enriching cryopreservation media with antioxidants, both enzymatic and non-enzymatic, resulting in enhanced post-thaw sperm viability and functionality by mitigating detrimental structural and functional impacts (6).

Emerging literature suggests variability among species, with distinct subsets exhibiting differential cryotolerance capacities ("good freezers," "acceptable freezers," and "poor freezers"), despite their genetic merit and reproductive performance under natural or fresh semen insemination scenarios (7, 8). Nevertheless, cryopreservation presents inherent challenges, primarily due to sperm's susceptibility to temperature fluctuations, compromising post-thaw viability (9).

ARTIFICIAL INSEMINATION

Artificial insemination represents the initial and most straightforward technique for augmenting productivity and has demonstrated efficacy across livestock species such as cattle, buffalo, sheep, and goats. The advent of artificial insemination using frozen-thawed doses has profoundly transformed the animal breeding sector since its inception in the 20th century (2). This method has been instrumental in advancing the genetic advancement of buffalo and cattle populations (10). Through artificial insemination, the superior genetic material from high-quality bulls is extensively leveraged, facilitating the insemination of a significant number of buffaloes and cattle with a single ejaculate (11). The integration of cryopreservation and artificial insemination has accelerated advancements in buffalo and cattle production and phenotypic traits at previously unparalleled rates (12). Additionally, this approach mitigates the dissemination of venereal diseases. Notably, a critical determinant contributing to sub-fertility in buffalo and cattle production stems from the utilization of inferior semen quality in artificial insemination procedures (13). Sub-fertility manifestations may arise due to disruptions in semen production, encompassing diminished or complete loss of sperm motility due to cryopreservation insults (14).

CRYOPRESERVATION

Cryopreservation serves as a pivotal technique in artificial insemination, facilitating the global dissemination of elite genetic material (15). The success of artificial insemination is notably influenced by the efficacy of semen cryopreservation (16). It is defined as the preservation of biological materials, including animal cells, plants, and genetic materials such as semen at -196 °C in liquid nitrogen. This method reduces metabolic activity, preserving cellular organelles and maintaining physiological, biological, and morphological functionalities (17, 18). The merits of frozen semen include indefinite storage capability, on-demand collection, and utilization flexibility (19). Among livestock, buffalo and cattle semen cryopreservation predominates the other species (20). This technology enables the long-term preservation of high-quality bull semen at

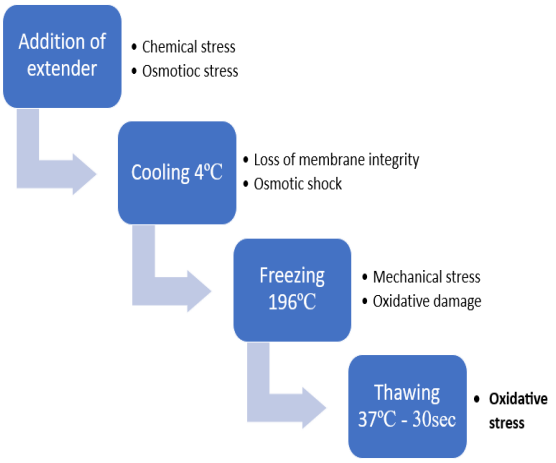

Figure 1. Cryopreservation steps and related stresses.

cryogenic temperatures (21). However, cryopreservation introduces challenges (Figure 1) such as osmotic stress elevation, pH fluctuations, potential ice crystal formation. These factors culminate in semen quality deterioration, lipid peroxidation, and alterations in the cholesterol-phospholipid ratio, leading to reactive oxygen species generation and oxidative stress in spermatozoa (22). Despite natural antioxidant defences in sperm membranes and seminal plasma, enzymatic deficiencies (such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase) can exacerbate damage during cryopreservation (23). During this time, the sperm plasma membrane transitions, rendering it vulnerable to environmental factors, compromising sperm capacitation and fertility (24, 25). Furthermore, extracellular ice crystal formation prompts solute concentration increases, leading to cellular dehydration, membrane damage, and reduced sperm viability, motility, and fertility (26, 27). Optimal cryopreservation media composition is crucial for preserving post-thaw semen quality (28, 29, 30). Ongoing research aims to develop cost-effective, efficient extenders to enhance sperm fertility and mitigate cryopreservation-induced damage (31). Approximately 40-50% of sperm may not survive cryopreservation even with optimized protocols, highlighting the inferiority of post-thaw spermatozoa compared to fresh semen (32, 33, 34, 35). Adverse cryopreservation effects encompass increased reactive oxygen species production, DNA fragmentation, and membrane phospholipid structure disruptions, affecting sperm viability and function (31, 36). Given the critical importance of sperm structure elements like the plasma membrane, acrosome, mitochondria, and chromatin for fertility, extensive research endeavors aim to mitigate cryopreservation-induced damages (37, 38). Strategies include offensive and defensive approaches, with semen extender supplementation emerging as a promising intervention (39, 40).

CHALLENGES ENCOUNTERED IN THE CRYOPRESERVATION OF BUFFALO SPERMATOZOA

Buffalo spermatozoa exhibit greater susceptibility to damage during cryopreservation than cattle spermatozoa, leading to diminished fertility rates following thawing. The cryopreservation and subsequent thawing processes adversely impact key cellular elements in buffalo spermatozoa, including the motility apparatus, plasma membrane, and acrosomal cap, accompanied by the leakage of intracellular enzymes (41). Despite the long-standing practice of artificial insemination in buffaloes, the conception rate lags, standing at approximately 30%, a notable contrast to cattle. The diminished freezability of buffalo semen can be attributed to distinct lipid compositions in the plasma membrane compared to cattle, rendering it more susceptible to damage during cryopreservation (23). Specifically, the plasma membrane of buffalo spermatozoa exhibits elevated concentrations of polyunsaturated fatty acids, notably arachidonic and docosahexaenoic acids. This lipid composition increases susceptibility to peroxidative damage induced by reactive oxygen species (ROS), resulting in compromised sperm functionality when juxtaposed with cattle sperm (42, 43).

REACTIVE OXYGEN SPECIES

At normal concentrations they a play role in sperm physiology but at higher concentrations they act as sperm pathology as depicted in Figure 2. Cryopreservation processes can induce the generation of reactive oxygen species (ROS), leading to detrimental interactions with the sperm cell plasma membrane, subsequently affecting fertility (44).

The freeze/thaw cycle further amplifies ROS production, compromising sperm functionality and contributing to spermatozoa degradation (45). Elevated ROS levels, coupled with diminished spermatozoa defence mechanisms, can induce damage not only to the plasma membrane but also to DNA integrity, thereby impairing sperm fertility and embryonic development potential (44). Notably, reduced motility of cryopreserved sperm post-thawing can be attributed to altered plasma membrane stability, increased membrane ion permeability, and elevated ROS production (46, 47). This decline in motility primarily stems from decreased adenosine triphosphate (ATP) production (48). While bovine bull semen possesses innate defences against ROS, these defences become insufficient during the critical freeze-thaw stress phase (49).

Cryopreservation and semen storage further modify sperm mitochondrial membrane integrity and resident electron transport chains, leading to excessive ROS production, including hydrogen peroxide (H_2O_2), nitric oxide (NO), and superoxide anion (O^{2-}). These alterations influence sperm capacitation and acrosome reactions (50, 51). Intriguingly, although ROS can be detrimental in excess, they also serve as essential mediators for normal sperm functions when present in controlled amounts (52, 53).

Spermatozoa possess three distinct membrane types: plasma, mitochondrial, and acrosomal. Each is rich in polyunsaturated fatty acids, rendering them highly susceptible to oxidative stress, particularly during cryopreservation (54). Continual lipid peroxidation chain reactions occur autonomously, with each reaction generating new ROS, ultimately culminating in comprehensive plasma membrane damage to spermatozoa (55).

OXIDATIVE STRESS

The literature underscores that the sperm membrane predominantly serves as the primary target for reactive oxygen species (ROS), with lipids representing potential targets within this context (33). Oxidative stress can impact virtually all cellular components, including lipids, proteins, nucleic acids, and carbohydrates, making them susceptible to oxidative damage (56). Lipid peroxidation of the plasma membrane triggers an influx of calcium and bicarbonate ions, thereby influencing sperm-oocyte fusion dynamics (57). Prolonged lipid peroxidation can further compromise the structural integrity of lipid matrices by disrupting double bonds. Consequently, this instability leads to functional disturbances in cell membranes, culminating in reduced sperm membrane fluidity (58). Moreover, seminal plasma's inadequate antioxidant levels can precipitate oxidative stress (OS), posing detrimental effects on

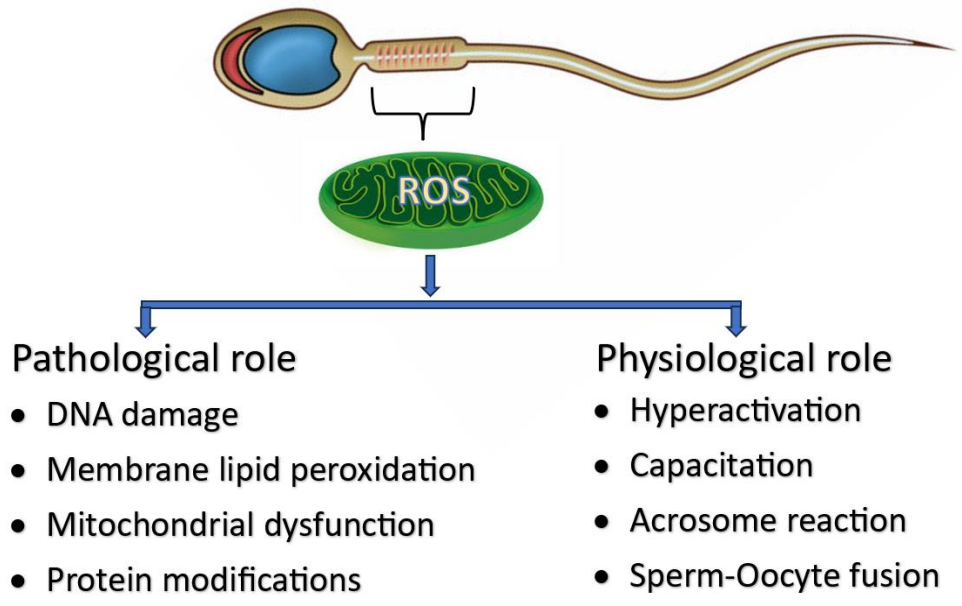

Figure 2. Roles of reactive oxygen species (OS) in sperm pathology and physiology.

spermatozoa (59).

PROTEIN ALTERATIONS IN SPERMATOZOA AS A RESULT OF REACTIVE OXYGEN SPECIES

Significant redox-dependent protein alterations such as thiol oxidation (60), tyrosine nitration (61), and S glutathionylation (61) have been linked to sperm dysfunction and changes in the paternal genome thus resulting in infertility, as shown in Figure 3.

S-glutathionylation modifies protein function, interactions, and localization in a variety of physiological processes and in pathological function. Protein S-glutathionylation is a post-translational modification that happens both under normal and oxidative stress circumstances (61). Glutathione containing spermatozoa provide a protective mechanism against oxidative damage caused by H_2O_2. Protein S-glutathionylation only occurs when reduced glutathione (GSH) react with SH group and resulting in enzyme inactivation. Sulfenic, sulfinic, and sulfonic acids are formed when cysteine residues in proteins are oxidised. Protein sulfenic and sulfinic acids can be reduced or conjugated to GSH to generate S-glutathionylated proteins, either enzymatically or non-enzymatically, using glutathione S-transferases, glutaredoxins (Grx). Higher quantities of glutathionylated proteins were found in spermatozoa treated with H_2O_2 than in untreated controls (61). Glutathione peroxidases carry out GSH's antioxidant activities, reducing H_2O_2 and lipid hydroperoxides by oxidizing GSH to oxidized glutathione (GSSG) (62). ROS and modulators such as glutaredoxins and glutathione transferases can target cysteine thiols (-SH) of proteins in particular. Protein glutathionylation is thought to be a protective process against oxidative stress. Non-enzymatic and enzymatic reactions can both lead to glutathionylation. The availability of GSH/GSSG is required for non-enzymatic glutathionylation. Glutathionylation in target proteins can be easily reversed by releasing GSH from cysteine residues (63).

S-nitrosylation and tyrosine nitration

The majority of nitrosative protein is formed by nitration and nitrosylation of the side chains of tyrosine, tryptophan, methionine, and

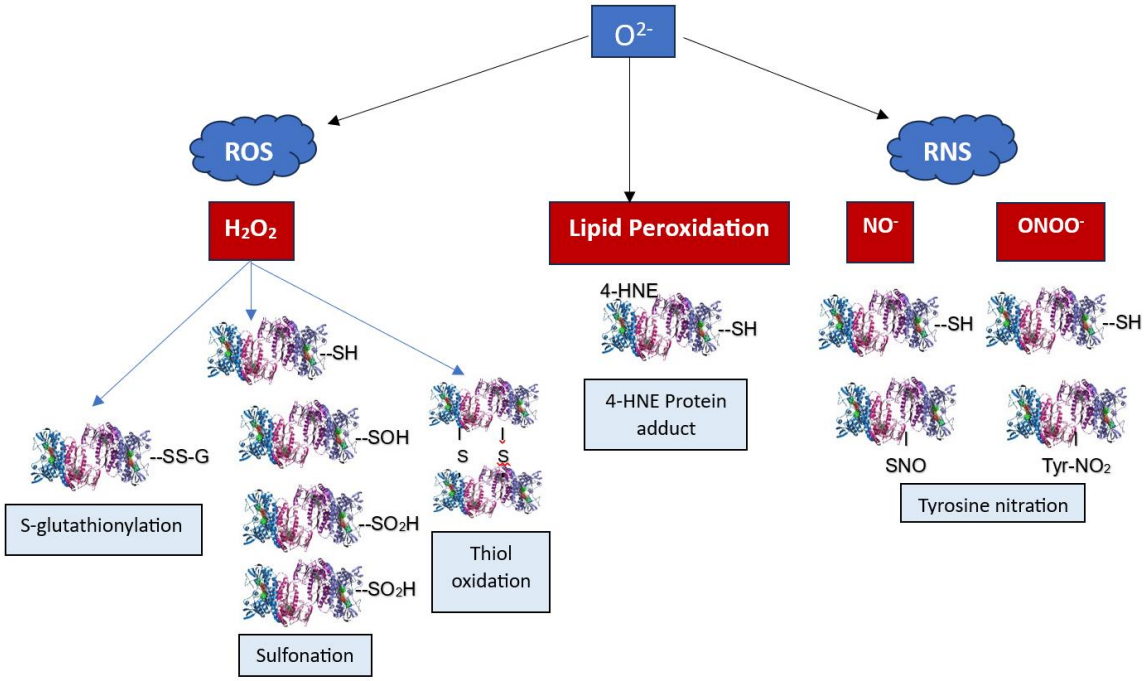

Figure 3. Redox-dependent protein modifications.

cysteine. Excess or unregulated nitric oxide reacts with ROS, resulting in an increase in reactive nitrogen species (64). 3-nitrotyrosine in proteins is a byproduct of the reactions of nitric oxide-derived oxidants, which are commonly linked with oxidative stress (65). Nitrogen oxygen species cause tyrosine nitration, a protein alteration. High concentrations of various ROS cause an increase in tyrosine nitration in spermatozoa, as evidenced by the use of peroxides (H_2O_2 or tert-BHP) and DA NONOate (NO donor) to produce a dose-dependent increase in S-glutathionylation and tyrosine nitration (66).

SPERM DNA DAMAGE

Hydrogen peroxide (H_2O_2) represents a reactive oxygen species (ROS) spontaneously generated within sperm. Aromatic amino acid oxidase (AAAO) and superoxide dismutase (SOD) reactions primarily contribute to H_2O_2 production in bovine spermatozoa (Figure 4). The mitochondria's electron transport chain (ETC) and the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) pathway serve as pivotal sites for ROS generation. Given its inherent reactivity, H_2O_2 can traverse cellular membranes with ease. Upon encountering Fe^{2+}, H_2O_2 catalyzes the formation of the hydroxyl radical (OH•) via the Fenton reaction. This OH• radical can impair mitochondrial mRNA synthesis by targeting mitochondrial DNA (mtDNA), consequently disrupting the synthesis of essential mitochondrial proteins vital for ETC and ATP production. Moreover, H_2O_2 interaction with nuclear DNA can instigate single-strand breaks (SSBs) and induce base damage within sperm DNA. Consistent with this perspective, research findings suggest that elevated intracellular H_2O_2 concentrations correlate with pronounced DNA damage in cryopreserved bovine sperm. Consequently, strategies aimed at curtailing H_2O_2 synthesis may enhance the DNA integrity of cryopreserved bovine sperm (115).

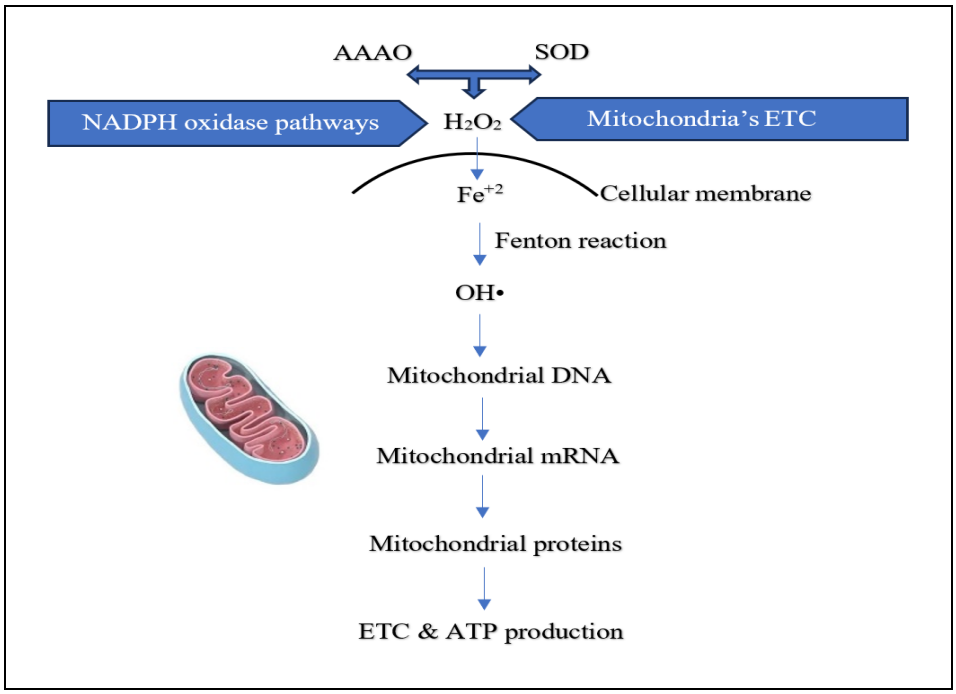

Figure 4. Effects of H_2O_2 on cellular biochemistry.

Table 1. Various semen additives used in the cryopreservation of buffalo semen.

Semen additive	Extender	Effective concentration	Breed	Result	Ref
Butylated hydroxy toluene (BHT)	Egg-yolk-tris-glycerol (EYTG)	0.5 mM BHT	Murrah	Increase in post-thaw motility, viability, HOST response, acrosome integrity	(23)
Cholesterol loaded cyclodextrins	Tris egg yolk glucose extender	5 mM	Egyptian water buffalo	Increased the functional competence of buffalo spermatozoa by increasing both post thaw motility and viability index and by decreasing the acrosomal defects	(75)
Coconut water	Tris-citric acid extender	20% coconut water (replaces 20% whole egg yolk)	Buffalo bulls	Increased progressive motility, live sperm, sperm abnormality, intact sperm acrosome and plasma membrane integrity	(76)
Cysteine HCl and ascorbic acid	Tris-fructose-egg yolk-glycerol extender	1 mg/mL and 0.2 mg/mL	Mehsana	Improvement in motility and viability of spermatozoa with lesser abnormality and reduced leakage of enzymes such as AST, LDH and AKP	(77)
Cysteine and taurine	Tris citrate fructose egg yolk glycerol (TFYG) extender	4 mg/mL taurine and 1 mg/mL cysteine	Surti	Enhanced progressive sperm motility, viability, and membrane integrity with reduced sperm/acrosome abnormalities	(78)
Garlic extract (*Allium sativum*)	Tris egg yolk based extender	3, 6, and 9 %	Buffalo bull	Increased -glucosidase activity, lower MDA levels and increased SOD and GPx activities, protect sperm from the detrimental effects of ROS, enhance sperm quality and energy source during sperm storage	(79)
Lemon, onion and garlic extract (LOG)	Tris egg yolk glycerol glucose	30 and 50 µl/mL LOG extract	Buffalo bull	Elevated antioxidant capacity and decrease in lipid peroxidation	(80)
Leptin and melatonin	Tris- egg yolk extender	20 ng/mL leptin and 10^{-3} M melatonin	Egyptian buffalo	Increased motility, livability and normality of buffalo spermatozoa	(81)
L-proline (Lp) and fulvic acid (FA)	Tris-egg yolk-based semen extender	40 mM Lp and 1.7% FA	Iranian water buffalo	Improves sperm motility, viability, Plasma membrane functionality and has antioxidant properties against ROS and free radicals	(82)
Quercetin	Tris citric acid extender	150 and 200 µM	Nili Ravi	Sperm progressive motility, plasma membrane integrity, supra vital plasma membrane integrity and acrosome integrity Sperm DNA integrity	(83)
Quercetin	OptiXcell Extender	10 µM	Egyptian Buffalo	Enhanced sperm motility, velocity, viability, membrane integrity	(84)
Sericin	AndroMed®	0.25–0.5% sericin	Murrah	Improved post thaw motility, membrane integrity and antioxidant status. improved frozen–thawed semen quality by preventing oxidative stress	(24)
Sodium alginate	Tris egg yolk based extender	0.4 mg/mL sodium alginate	Murrah	Protects sperm by: a) preventing generation of free radicals, b) scavenging the excess free radicals, c) chelating heavy metals, d) maintaining sperm plasma membrane fluidity, g) potentiating the antibacterial capacity of the semen extender	(57)
Taurine and trehalose	Egg yolk tris citrate (EYTC) extender	Taurine (50 mM) and trehalose (100 mM),	Murrah	Post-thaw motility, viability and membrane integrity, decreased cryo-capacitation and tyrosine phosphorylation	(85)
Trehalose	Tris-fructose-egg ylk-citrate extender	100 mM	Surti	Improvement in post-thaw semen quality in terms of progressive sperm motility, sperm viability, HOST response and reduced sperm abnormalities	(86)

SEMEN ADDITIVES

Semen additives refer to supplementary agents incorporated into semen extenders with the objective of augmenting semen longevity. These additives predominantly exhibit antioxidative attributes, facilitating the scavenging and neutralization of free radicals, thereby shielding sperm cells from lipid peroxidative impairment. Beyond their antioxidative capabilities, certain additives enhance parameters such as sperm motility, stability, and overall fertility. Recent advancements in research have led to the development of novel additives aimed at safeguarding male gametes from detrimental effects associated with cryopreservation (67). The incorporation of semen additives extends the longevity of semen without inducing adverse effects, concurrently fortifying the integrity of spermatozoal membranes and inhibiting ice crystal formation. While seminal fluid inherently contains various antioxidant entities, including catalase, glutathione peroxidase, superoxide dismutase, and reduced glutathione, designed to counteract reactive oxygen species (ROS) under physiological conditions, the intrinsic antioxidant defences of spermatozoa remain relatively fragile. Consequently, germ cells exhibit increased vulnerability to oxidative stress (99).

Antioxidants

Tables 1 and 2 include a meticulous analysis and documentation of studies conducted to elucidate the properties and applications of antioxidants in sperm biology. This detailed examination encompasses a range of critical parameters, including specific experimental outcomes associated with each antioxidant, the precise concentrations at which they were administered, the durations or years over which they were utilized, and the specific types of extenders employed in conjunction with these antioxidants. Furthermore, this comprehensive data presentation is specifically tailored to provide insights and understanding related to their effectiveness and application nuances in both buffalo and cattle contexts.

Antioxidants play a pivotal role in safeguarding spermatozoa against oxidative damage, which arises from an imbalance characterized by elevated levels of oxidizing agents within the spermatozoa (23). Strengthening semen diluents with appropriate antioxidant supplements becomes imperative to mitigate reactive oxygen species (ROS) induced damages during the freeze-thawing process of bull semen (68 Khan I et al., 2021). The seminal plasma inherently harbors an intricate antioxidant system, crucial for shielding sperm from detrimental effects attributable to ROS (69). Nevertheless, the endogenous antioxidant capacity intrinsic to spermatozoa remains insufficiently robust compared to somatic cells, rendering them susceptible to oxidative stress (70). A plethora of investigations have been conducted concerning the incorporation of diverse antioxidants into extenders to safeguard spermatozoa against ROS-induced impairments. The integration of exogenous antioxidants into extenders has been substantiated as beneficial in preserving sperm quality (71, 72). The limited endogenous antioxidant reserves in spermatozoa fall short of providing adequate protection against the elevated ROS production ensuing from cryopreservation procedures (73 Mazzilli et al., 1995).

Antioxidants previously evaluated primarily exerted their effects through direct or indirect scavenging of free radicals (74). Moreover, beyond free radical scavenging, there exists a requisite for additives possessing metal-reducing properties, given that metals act as catalysts in oxidative reactions. Redox-active metals such as iron, copper, and chromium engage in redox cycling, while redox-inactive metals, such as lead, cadmium, and mercury, deplete primary antioxidants and enzymes within cells (87). Notably, hen egg yolk serves as a principal component in extenders for bovine semen, potentially introducing both redox-active and redox-inactive metals that may augment ROS production. Consequently, the chelation of undesirable metals within semen extenders emerges as a critical imperative (57). Contrastingly, plant-derived extracts offer natural antioxidants characterized by diminished cytotoxicity relative to synthetic counterparts (88).

Various semen additives

Butylated hydroxy toluene (BHT) serves as a lipid-soluble antioxidant and is a synthetic derivative analogous to Vitamin A. When administered at optimal concentrations, it enhances sperm cell motility and viability in specific breeds like the Holstein and Sahiwal. Due to its lipid-soluble nature, BHT operates as an antioxidant both intracellularly and extracellularly, facilitating its integration into the sperm membrane. This incorporation augments membrane fluidity, mitigating intracellular ice-crystal nucleation and thereby safeguarding sperm integrity. Additionally, BHT exhibits scavenging properties against reactive oxygen species (ROS) in the sperm's vicinity, converting them into less detrimental hydroperoxides, consequently attenuating ROS-induced damage during cryopreservation processes (89).

Cysteine serves as a notable agent recognized for its role as an intracellular antioxidant, shielding cells from oxidative stress-induced damage as documented by Meister and Anderssson (90). Incorporating cysteine into the semen diluent has

Table 2. Various semen additives used in the cryopreservation of cattle semen.

Semen additive	Extender	Effective concentration	Breed	Result	Ref
N-acetyl-L-cysteine	Tris-citric acid-egg yolk extender	1.0 mM	Local Friesan	Increased post-thaw motility, viability and maintains acrosomal integrity of cryopreserved local Friesian bull's spermatozoa	(107)
N-acetyl cysteine	Tris-yolk fructose (TYF) extender	2 and 4 mM NAC	Holstein	Increased post-thaw motility, viability, plasma membrane integrity and acrosome integrity	(108)
Ascorbic acid	Tris-egg-yolk-citric-acid-fructose-glycerol (TEYCAFG) extender	5 mM	Cross-bred cattle bull	Increased live spermatozoa, acrosomal integrity and HOST-positive spermatozoa,	(109)
Butylated hydroxytoluene (BHT)	Lecithin based Bioxcell® (BX) and Egg yolk tris citrate (EYTC)	0.5 mM/mL BX and 1–1.5 mM/mL BHT for EYTC	Crossbred bulls	decreases MDA concentrations, improvement of frozen–thawed bull sperm quality and protects against DNA damage by reducing oxidative stress, increased post-thaw motility, acrosome integrity, DNA integrity	(89)
Chloroquine diphosphate and ascorbic acid	Tris-based extender	Chloroquine diphosphate and ascorbic acid were 10^{-5} M and 0.02%	Jersey	Protection for acrosome during post-thaw incubation and significant improvement in progressive motility, live sperm percentage, reaction to hypo-osmotic solution and acrosomal integrity	(110)
Curcumin	Tris-egg yolk-based semen extender	25 µM	Kankrej	Reduction in lipid peroxidation, SOD activity and enzyme activity	(99)
L-cysteine and vitamin E	Egg- Yolk-Tris-Glycerol (EYTG)	7.5 mmol L-cysteine and 4.8 mmol vitamin E	Crossbred groups (Sahiwal × Holstein-Friesian) and (Achai × Jersey	Increase in post-thaw motility, membrane functionality, acrosomal integrity,viability percentage, DNA integrity and mitochondrial membrane potential	(68)
Genistein	Skim milk – soy lecithin extender	1 mmol and 2 mmol	Ongole	Higher motility, viability, and membrane integrity and acrosome integrity	(111)
Green tea extract	Tris-based egg yolk extender	0.15 mg into 100 mL tris-based egg yolk extender	Bali bulls (*Bos sondaicus*)	Improved post-thaw motility, viability, plasma membrane integrity of Bali bull sperm	(40)
Honey (*Apis mellifera*)	Bioxcell Extender	1% concentration of honey	Jersey	Increased progressive motility, and liveability of sperm	(98)

Table 2 continued. Various semen additives used in the cryopreservation of cattle semen.

Semen additive	Extender	Effective concentration	Breed	Result	Ref
Iodixanol	Tris egg yolk	2.5%	*Bos indicus* [Thai native bull]	Improved frozen semen quality and progressive motility	(112)
Low-density lipoprotein (LDL)	AndroMed® extender	8% LDL supplementation	Holstein	Higher proportions of plasma membrane-intact sperm, acrosome-intact sperm, and spermatozoa showing high mitochondrial potential	(113)
Lycopene	Tris egg yolk citrate extender	1.5 mmol/L	Simmental	Improves structural stability, activity,and oxidative profile of bovine gametes and improves semen handling and storage protocols in cattle breeding	(100)
Pomegranate juice	Tris-citric acid-egg yolk-fructose extender	10%	Cattle bull	Improved post-thaw sperm motility, membrane integrity and viability and decreased total sperm abnormalities of cattle cryopreserved semen.	(102)
Quercetin	Tris citrate fructose egg yolk glycerol (TFYG) extender	82.7 µmol / L (25 µg / mL)	Holstein	Positive effects on DNA integrity.	(114)
Sodium pyruvate	Triladyl extender	5 mM	Simmental	Improved motility, plasma membrane and acrosome integrity, mitochondrial membrane potential and DNA integrity of cryopreserved bovine sperm	(115)
Taurine	Andromed extender	50 mM	Gir	Increased glutathione reductase levels and decreased lipid peroxidation	(116)
Taurine and trehalose	Tris–egg yolk citrate (EYTC) extender	50 mM taurine or 100 mM trehalose	Crossbreed cattle “Karan Fries” (Holstein Friesian × Tharparkar)	Improvement in the fertilizing capacity of cryopreserved Karan Fries spermatozoa	(25)
Taurine and trehalose	Egg yolk tris citrate (EYTC) extender	Taurine (50 mM) and trehalose (100 mM),	Karan Fries	Post-thaw motility, viability and membrane integrity, decreased cryo-capacitation and tyrosine phosphorylation in both buffalo and cattle bulls	(85)
Vitamin C with catalase and vitamin C with reduced glutathione	Tris fructose egg yolk glycerol extender	2.5 mM vitamin C , 100 IU/mL catalsae, 2 mM reduced glutathione	Holstein	Increased post-thaw motility, Viability, Plasma membrane integrity, Acrosome integrity	(117)

demonstrated efficacy in enhancing both freeze-thawed motility and viability across various species, including boar in 2010 (91), buck in the same year (92) and bull semen in 2009 (93). Serving as a precursor to intracellular glutathione, cysteine has demonstrated the ability to readily traverse cellular membranes, thereby augmenting intracellular GSH biosynthesis both in vivo and in vitro. This facilitates the protection of membrane lipids and proteins owing to its inherent indirect radical scavenging properties. Moreover, Memon et al. in 2011 highlighted cysteine's cryoprotective attributes, specifically enhancing the functional integrity of acrosome and mitochondria, thereby elevating post-thawed sperm motility across multiple species (94). L-cysteine is characterized by its pivotal thiol group, functioning as a non-enzymatic antioxidant, with a notable capability to permeate sperm cells, as elucidated by Khan et al. in 2021 (68).

Trehalose, a non-permeating disaccharide, functions as a hypertonic medium, facilitating cellular osmotic dehydration prior to freezing and thereby reducing cellular injury caused by crystallization as elucidated by Bucak et al. in 2007 (95). Moreover, Kumar et al. in 2023 highlighted its role as an enzymatic scavenger (86). Through its osmotic properties, trehalose exerts protective effects against oxidative

damage, playing a pivotal role in safeguarding spermatozoa from ROS detriment.

Honey, derived from *Apis mellifera* bee, is abundant in phenolic compounds, exhibiting a robust correlation with antioxidant activity (96). In 2004, Fuller (97) detailed the composition of honey, highlighting its high content of carbohydrates, proteins, amino acids, vitamins, minerals, and antioxidants, effectively thwarting free radical activities. Moreover, honey's rich glucose and fructose content aids in maintaining diluent osmotic pressure, inducing cellular dehydration, and mitigating ice crystal formation in and around spermatozoa. This attributes honey as a valuable additive in frozen semen (29, 98).

Curcumin, chemically known as 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione (CUR), is a bioactive constituent extracted from the tropical rhizome of the Zingiberaceae family, specifically turmeric (99). Studies have demonstrated that curcumin mitigates the adverse effects of cryopreservation on both spermatozoa and stem cells, as evidenced by research conducted by Tvrda et al. (100) and Navjot et al. (101).

Pomegranate juice is recognized for its potent antioxidant properties, both in vivo and in vitro. This juice is notably rich in vitamin C and various polyphenolic compounds such as anthocyanins, punicalagin, ellagic acid, and gallic acid, as highlighted by Sheshtawy et al. (102). Additionally in 2002, Halvorsen et al. emphasized the elevated antioxidant concentrations present in pomegranate fruit, quantified at 11.33 mmol/100 g (103).

Lemon, onion, and garlic extracts, which exhibit significant antioxidant capacities, have been effectively utilized to diminish intracellular ROS levels. These extracts provide protection against oxidative damage to cellular components including lipids, DNA, and mitochondrial structures, as supported by studies from Giampieri et al. (104) and Brito et al. (105).

Green Tea Extract (GTE) is replete with polyphenols, notably catechins, renowned for their antioxidant capabilities. Particularly, it is abundant in (-)-epigallocatechin-3-gallate (EGCG), which either directly inhibits the generation of reactive oxygen species (ROS) or indirectly bolsters the endogenous defence mechanisms, as elucidated by Park and Yu (106). Furthermore, GTE supplementation is beneficial due to its provision of essential vitamins including A, B1, B3, B5, C, E, and K. Notably, vitamins C and K function as antioxidants, counteracting free radicals and safeguarding cellular membrane integrity against lipid peroxidation, as delineated by Khan H et al. (118) Park and Yu (119).

Coconut water, recognized as a non-pathological fluid devoid of contaminants and toxins, possesses a myriad of health benefits including antiviral, antibacterial, antifungal, antiparasitic, and antioxidant properties. Its beneficial impact on semen quality can be attributed to its rich composition of free sugars, antioxidants, and essential minerals. Consequently, coconut water has found successful application in the context of bovine embryo freezing and culture, as corroborated by Soltan et al. (76).

Lycopene, a natural carotenoid abundant in certain fruits, has garnered attention for its antioxidative properties. Research elucidated by Tvrda et al. underscores lycopene's potential in augmenting sperm motility, viability, morphology, and testicular oxidative equilibrium post-exposure to medications (120).

Genistein, a phytoestrogen present in soybean plants, exhibits multifaceted functionalities including inhibition, angiogenesis, fat peroxidation, antioxidant, and anti-cancer activities. In 2010, Martinez-soto et al. highlighted its antioxidant and anti-inflammatory attributes, demonstrating its capacity to modify hemodialysis membranes and significantly reduce ROS (121). Consequently, genistein exerts a direct impact on the functionality of mature sperm cells, potentially bolstering the overall reproductive process (111).

Sodium alginates, derived from brown seaweed, are inherently anionic polysaccharides characterized by their biocompatibility, non-immunogenicity, and non-toxic nature. Numerous studies have substantiated their richness in antioxidant compounds, capabilities as transition metal chelators, and their inherent antibacterial, antiviral, and antifungal properties. Kumar et al. conducted an evaluation of sodium alginate's potential for sperm encapsulation, concluding that this natural polymer exhibits non-toxic properties towards sperm cells (57).

Sericin, derived from the silkworm *Bombyx mori*, is a water-soluble globular protein characterized by a composition rich in amino acids such as aspartic acid and serine. Notably,

sericin exhibits pronounced protective effects against lipid peroxidation, mitigating cellular demise and safeguarding against various stressors, as evidenced by Kumar et al. (24).

Fulvic acids, derived from peat, are organic compounds revered for their antioxidative properties and solubility in diverse solutions. These compounds have been associated with improved semen quality under heat stress conditions and cryoprotective effects on goat buck semen, as evidenced by Ramazani et al. (82) and Xiao et al. (122).

Ascorbic acid, commonly known as Vitamin C, is an intrinsic antioxidant present within the epididymal fluid and seminal plasma across various species. Its primary role involves safeguarding sperm from ROS (110). Specifically, Vitamin C diminishes oxygen radicals, neutralizes existing ROS, rejuvenates additional antioxidant systems, and upholds the genetic stability of sperm cells by thwarting oxidative harm to sperm DNA, as outlined by Singh et al. (109). Furthermore, the incorporation of Vitamin C into an extender can enhance sperm functionality by mitigating cellular damage through its persistent radical-neutralizing capabilities, as highlighted by Anane and Creppy et al. in 2001 (123).

Vitamin E emerges as a paramount component within the antioxidant defence mechanism of spermatozoa, predominantly shielding the cellular membrane against ROS-induced lipid peroxidation (LPO), as substantiated by Yousef et al. (124).

Glutathione, a naturally occurring tripeptide within semen, serves as a pivotal intracellular defence mechanism against oxidative stress elicited during semen cryopreservation and the subsequent freeze-thaw processes. Notably, post-cryopreservation analyses revealed diminished glutathione concentrations in bull semen. Augmenting the Tris extender with glutathione markedly enhanced various sperm attributes including motility, viability, membrane integrity, acrosome preservation, while concurrently diminishing malondialdehyde (MDA) levels, as elucidated by Eidan (117).

Catalase, an enzymatic antioxidant ubiquitous in sperm and seminal plasma, plays a pivotal role in mitigating ROS-induced damage. Eidan elucidated the benefits of catalase supplementation in enhancing sperm viability and reducing malondialdehyde concentrations (117).

Sodium pyruvate functions as a potent scavenger for hydrogen peroxide (H_2O_2), enzymatically decarboxylating it into acetic acid, carbon dioxide, and water, devoid of any oxygen liberation. Distinguished from alternative antioxidants, pyruvate culminates as the terminal product within glycolysis during cellular metabolism, subsequently converting into acetyl-CoA, which fuels the Krebs cycle to generate ATP via oxidative phosphorylation. Given that superoxide anions (O_2-) are the predominant ROS within spermatozoa, their intricate interactions, including self-dismutation, culminate in H_2O_2 generation. Consequently, even minor variations in oxygen levels could significantly influence ROS production and subsequent sperm cell viability. In this context, sodium pyruvate emerges as a preferred antioxidant due to its multifaceted benefits encompassing intracellular energy metabolism augmentation, post-thaw sperm quality enhancement, and its capacity to neutralize H_2O_2 without inducing oxygen release. Existing literature underscores the ability of exogenous pyruvate to amplify mitochondrial activity and post-thaw sperm motility, as affirmed by Korkmaz et al. (115).

Cholesterol-loaded cyclodextrins (CLC) supplementation to semen extenders has been associated with enhanced quality of cryopreserved spermatozoa. This improvement is attributed to the stabilization of spermatozoan plasma and mitochondrial membranes, coupled with a notable reduction in DNA damage, as indicated by Abouelezza et al. (75).

Low-density lipoproteins (LDLs) derived from egg yolk represent a crucial component, renowned for its cryoprotective capabilities as outlined in 2016 by Simonik et al. (125). These LDLs have been observed to positively influence spermatozoa structure and extracellular conditions, as shown by Hu et al. (126). However, the utilization of whole egg yolk presents challenges such as inconsistent microscopic evaluations, potential inter-species protein interactions, standardization issues, and susceptibility to microbial contamination. Consequently, non-animal-derived alternatives, including soybean lecithin or liposomes, have gained prominence. Incorporating the LDL fraction into a soybean lecithin-based extender may offer an effective strategy to enhance the quality of bull insemination doses, with synergistic cryoprotective benefits established in

prior studies by Beran et al. (127) and Stadnik et al. (128).

Leptin, primarily recognized for its role in energy homeostasis, has emerged as a modulator of sperm metabolism and motility. Studies by Jorsaraei et al. in 2008 (129) and Khaki et al. in 2013 (130) elucidate leptin's significance in preserving sperm viability and motility, particularly in buffalo semen subjected to cooling.

Melatonin, an endogenous compound, exhibits potent antioxidant properties attributed to its efficacy in scavenging diverse reactive oxygen and nitrogen species. Khalek et al. substantiated melatonin's protective role against oxidative stress, emphasizing its relevance in mitigating cellular damage (81).

Iodixanol's protective mechanisms encompass the preservation of sperm membrane integrity and motility during freezing and thawing processes. This preservation is achieved through modulations in the glass transition temperature and alterations in ice crystal formation, facilitating water removal at lower temperatures, as highlighted by Chuawongboon et al. (112).

L-proline, an essential amino acid, possesses multifaceted functions encompassing osmoprotection, stabilization of cellular structures, and redox balance maintenance. Ramazani et al. highlight L-proline's antioxidant and osmoprotective attributes, particularly against freezing-induced detrimental effects across various species (82).

Quercetin, a natural flavonoid abundant in various foods such as berries, citrus fruits, tea, red wine, cocoa, and red onions, possesses significant antioxidant properties. Its polyphenolic structure enables it to act as a potent scavenger of free radicals, thereby inhibiting oxidation processes, as elucidated by El-Khawagah et al. in 2020 (84). Further research underscores quercetin's efficacy in enhancing sperm viability by mitigating oxidative stress and ROS damage post-thawing (83, 114, 131). Moreover, in synergy with alpha-tocopherol, quercetin curtails lipid peroxidation and upregulates the expression of critical enzymes like glutathione s-transferase and glucuronosyl transferase, as highlighted by Omur and Uluyol (132).

Taurine, an amino sulfonic acid prevalent in animal tissues, was first isolated from ox bile in 1827, followed by human bile in 1846, as documented by Marcinkiewicz and Kontny (133). This multifaceted compound serves diverse biological functions encompassing bile acid conjugation, antioxidative properties, osmoregulation, membrane stabilization, and signaling modulation, as delineated by Bouckenooghe et al. (134). Originating from cysteine, taurine is naturally present in fish, meat, and breast milk. Its role as an antioxidant entails neutralizing physiological hypochlorite and hypobromite toxicity, inhibiting lipid peroxidation, and safeguarding cells against ROS accumulation (135). Additionally, taurine's efficacy as a non-permeating sperm cryoprotectant has been demonstrated to mitigate cellular damage during cryopreservation (136).

N-acetyl-L-cysteine (NAC) emerges as a pivotal antioxidant with detoxifying attributes. Its antioxidative mechanisms operate at both intracellular and extracellular levels. Intracellularly, NAC serves as a precursor for glutathione (GSH) synthesis, facilitating cellular penetration and subsequent deacetylation to form L-cysteine (116), thereby bolstering GSH biosynthesis (137). Extracellularly, NAC directly combats oxidant radicals as a nucleophile and augments glutathione-S-transferase activity (77, 107). The robust antioxidant capabilities of NAC are instrumental in shielding sperm cells from formidable oxidative stress conditions, thereby mitigating potential oxidative damage, as highlighted by Perez et al. in 2015 (138).

CONCLUSION

In summary, our comprehensive review delineates the efficacy of diverse semen additives in mitigating oxidative stress and associated detriments during cryopreservation processes. Contemporary investigations have rigorously assessed these additives, either in isolation or combination, with a predominant focus on augmenting the post-thaw quality of buffalo and cattle semen. Notably, these additives exhibit antioxidative attributes that facilitate the scavenging and neutralization of free radicals, thereby enhancing seminal post-thaw characteristics. Nevertheless, further studies employing advanced methodologies such as Computer-Assisted Semen Analysis (CASA) and flow cytometry are imperative to elucidate the intricate molecular mechanisms and functional roles of these seminal additives

in optimizing the quality of frozen-thawed semen.

REFERENCES

1. Bailey J, Morrier A & Cormier N (2003) *Can. J. Anim. Sci*. **83(3)**, 393–401. https://doi.org/10.4141/A03-024
2. Lonergan P (2018) *Animal* **1**, 4–18.
3. Veerkamp RF & Beerda B (2007) *Theriogenology* **68(1)**, 266–273. https://doi.org/10.1016/j.theriogenology.2007.04.034
4. Fickel J, Wagener A & Ludwig A (2007) *Eur. J. Wildl. Res.* **53(2),** 81–89. https://doi.org/10.1007/s10344-007-0089-z
5. Kinkar KMD, Debajyoti S, Asish D, Mokidur R & Sangram SK (2020) *J. Entomol. Zool. Stud*. **8(6)**, 1493–1505.
6. Yanez-Ortiz I, Catalán J, Rodríguez-Gil J E, Miró J & Yeste M (2022) *Anim. Reprod. Sci*. **246,** 106904. https://doi.org/10.1016/j.anireprosci.2021.106904
7. Loomis PR & Graham JK (2008) *Anim. Reprod. Sci.* **105(1–2)**, 119–128. https://doi.org/10.1016/j.anireprosci.2007.11.010
8. Mazur P, Leibo SP & Chu EHY (1972) *Exp. Cell Res*. **71(2)**, 345–355.
9. Nijs M, Creemers E, Cox A, Janssen M, Vanheusden E, Castro-Sanchez YC, Thijs H & Ombelet W (2009) *Reprod. Biomed. Online* **19(2),** 202–206. https://doi.org/10.1016/s1472-6483(10)60073-9
10. Bishist R, Raina VS, Bhakat M, Mohanty T. K, Lone SA & Sinha R (2020) *Buffalo Bull*. **39(3)**, 337–344.
11. Hussain M, Begum SS, Kalita MK, Ahmed KU & Nath R (2018) *Int. J. Chem. Stud*., **6(5)**, 354–361.
12. Yangngam Y, Chapanya S, Vongpralub T, Boonkum W & Chankitisakul V (2021) *Czech J. Anim. Sci*. **66(1)**, 13–20. https://doi.org/10.17221/104/2020-CJAS
13. Gnoth C, Godehardt E, Frank-Herrmann P F, Friol K, Tigges J & Freundl G (2005) *Hum. Reprod*. **20(5),** 1144–1147. https://doi.org/10.1093/humrep/deh870
14. Dirami T, Rode B, Jollivet M, Da Silva N D, Escalier D, Gaitch N, Norez C, Tuffery P, Wolf JP, Becq F, Ray PF, Dulioust E, Gacon G, Bienvenu T & Touré A (2013) *Am. J. Hum. Genet.* **92(5),** 760–766. https://doi.org/10.1016/j.ajhg.2013.03.016
15. Trachootham D, Alexandre J & Huang P (2009) *Nat. Rev. Drug Discov*. **8(7)**, 579–591. https://doi.org/10.1038/nrd2803
16. Prasetyowati MH, Pradista LA, Widyas N & Prastowo S (2021) *IOP Conference Series: Earth and Environmental Science: International Conference on Livestock in Tropical Environment* **902(1)**, 012008, https://doi.org/10.1088/1755-1315/902/1/012008
17. Watson PF (2000) *Animal Reproduction Science* **60–61**, 481–492. https://doi.org/10.1016/s0378-4320(00)00099-3
18. Kostaman T & Setioko AR (2011) *Bulletien. Anim. Vet. Sci*., *Wartazoa* **21(3),** 145-152.
19. Sanocka D & Kurpisz M (2004) *Reprod. Biol. Endocrinol.* **2,** 12. https://doi.org/10.1186/1477-7827-2-12
20. Bhattacharyya HK, Goswami BK, Bujarbaruah KM, Deka BC & Biswas RK (2009) *Theriogenolog*y **72(5)**, 699–703. https://doi.org/10.1016/j.theriogenology.2009.05.010
21. Fakhrildin MBMR & Alsaadi RAR (2014) *J. Family. Reprod. Health* **8(1)**, 27–31.
22. Raheja N, Choudhary S, Grewal S, Sharma N & Kumar N (2018) *J. Entomol. Zool. Stud*., **6(3)**, 239–245.
23. Nain D, Mohanty TK, Dewry RK, Bhakat M, Nath S, Gupta VK & Parray MA (2023) *CryoLetters* **44(1)**, 57–65. https://doi.org/10.54680/fr23110110612
24. Kumar P, Kumar D, Sikka P & Singh P (2015) *Anim. Reprod. Sci*., **152**, 26–31. https://doi.org/10.1016/j.anireprosci.2014.11.015
25. Chhillar S, Singh VK, Kumar R & Atreja SK (2012) *Anim. Reprod. Sci*., **135(1–4)**, 1–7. https://doi.org/10.1016/j.anireprosci.2012.08.029
26. Stephens TD, Brooks RM, Carrington JL, Cheng L, Carrington AC, Porr CA & Splan RK (2013) *J. Equine Vet. Sci*. **33(8),** 615–621. https://doi.org/10.1016/j.jevs.2012.10.004
27. Bhakat M, Mohanty TK, Raina VS, Gupta AK, Pankaj PK, Mahapatra RK & Sarkar M (2011) *Asian-Australasian J. Anim. Sci*., **24(10)**, 1348–1357. https://doi.org/10.5713/ajas.2011.10243

28. Sheshtawy RIE, Sisy GAE & Nattat WSE (2015) *Asian. Pac. J. Reprod.*, **4(1)**, 26–31.
29. Yimer N, Muhammad N, Sarsaifi K, Rosnina Y, Wahid H, Khumran AM & Kaka A (2015) *Mal. J Anim. Sci.* **18**, 47–54.
30. Patel GK, Haque, N, Madhavatar M, Chaudhari AK, Patel DK, Bhalakiya N, Jamnesha N, Patel P & Kumar R (2017) *J. Pharmacogn. Phytochem.* **1**, 307–313.
31. Layek SS, Mohanty TK, Kumaresan A & Parks JE (2016) *Anim. Reprod. Sci*. **172,** 1–9. https://doi.org/10.1016/j.anireprosci.2016.04.013
32. Baust JG, Gao D & Baust JM (2009) *Organogenesis* **5(3),** 90–96. https://doi.org/10.4161/org.5.3.10021
33. Bansal AK & Bilaspuri GS (2011) *Veterinary Med. Int.* **2011,** 686137. https://doi.org/10.4061/2011/686137
34. Aisen E, Quintana M, Medina V, Morello H & Venturino A (2005) *Cryobiology* **50(3)**, 239–249. https://doi.org/10.1016/j.cryobiol.2005.02.002
35. Yoon SJ, Rahman MS, Kwon WS, Park YJ, & Pang MG (2016) *PLoS One* **11(3)**, e0152690. https://doi.org/10.1371/journal.pone.0152690
36. Makarevich A, Špaleková E, Kubovičová E, Bezdíček J & Chrenek P (2022) *Czech J. Anim. Sci.* **67(9),** 356–364. https://doi.org/10.17221/113/2022-CJAS
37. Khalil WA, Harairy MAE, Zeidan AEB, Hassan MAE & Elsaeed OM (2018) *Int. J. Vet. Sci. Med*., **6, Supplement, 2018**, S49-S56
38. Miguel-Jimenez S, Rivera Del Alamo MMRD, Álvarez-Rodríguez M, Hidalgo CO, Peña AI, Muiño R, Rodríguez-Gil JE & Mogas T (2020) *Anim. Reprod. Sci.* **215**, 106315. https://doi.org/10.1016/j.anireprosci.2020.106315
39. Hezavehei M, Sharafi M, Kouchesfahani H M, Henkel R, Agarwal A, Esmaeili V & Shahverdi A (2018) *Reprod. Biomed. Online* **37(3)**, 327–339. https://doi.org/10.1016/j.rbmo.2018.05.012
40. Prastiya RA, Suprayogi TW, Debora AE, Wijayanti A, Amalia A, Sulistyowati D & Nugroho AP (2023) *Anim. Biosci.* **36(2),** 209–217. https://doi.org/10.5713/ab.22.0184
41. Kadirvel G, Kumar S, Kumaresan A & Kathiravan P (2009) *Anim. Reprod. Sci*. **116(3–4)**, 244–253. https://doi.org/10.1016/j.anireprosci.2009.02.003
42. Lenzi A, Gandini L, Lombardo F, Picardo M, Maresca V, Panfili E, Tramer F, Boitani C & Dondero F (2002) *Contraception* **65(4),** 301–304. https://doi.org/10.1016/s0010-7824(02)00276-7
43. Ros-Santaella JL & Pintus E (2021) *Antioxidants* **10(5),** 772. https://doi.org/10.3390/antiox10050772
44. Nair SJ, Brar AS, Ahuja CS, Sangha SPS & Chaudhary KC (2006) *Anim. Reprod. Sci*. **96(1–2)**, 21–29. https://doi.org/10.1016/j.anireprosci.2005.11.002
45. Pons-Rejraji HP, Sion B, Saez F, Brugnon F, Janny L & Grizard G (2009*) Gynecol. Obstet. and Fertil*. **37(6)**, 529–535. https://doi.org/10.1016/j.gyobfe.2009.04.015
46. Bilodeau JF, Chatterjee S, Sirard MA & Gagnon C (2000) *Mol. Reprod. Dev*. **55(3),** 282–288. https://doi.org/10.1002/(SICI)1098-2795(200003)55:3<282::AID-MRD6>3.0.CO;2-7
47. Hafez ESE & Hafez B (2013) (Eds) *Reproduction in Farm Animals (7th ed)*, Lippincott Williams and Wilkins, Philadelphia, USA.
48. Awda BJ, Mackenzie-Bell MM & Buhr MM (2009) *Biol. Reprod*. **81(3),** 553–561. https://doi.org/10.1095/biolreprod.109.076471
49. Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ & Sikka SC (1999) *Free Radical Biol. Med*. **26(7–8)**, 869–880. https://doi.org/10.1016/s0891-5849(98)00275-5
50. Nichi M, Bols PEG, Züge RM, Barnabe VH, Goovaerts IGF, Barnabe RC & Cortada CNM (2006) *Theriogenology* **66(4)**, 822–828. https://doi.org/10.1016/j.theriogenology.2006.01.056
51. Leahy T & Gadella BM (2011) *Reproduction* **142(6),** 759–778. https://doi.org/10.1530/REP-11-0310
52. Liman MS, Hassen A, McGaw LJ, Sutovsky P & Holm DE (2022) *Animals* **12(9),** 1–12. https://doi.org/10.3390/ani12091130
53. Rath D, Bathgate, R., Rodriguez-Martinez, HR, Roca J, Strzezek J & Waberski D (2009) *Soc. Reprod. Fertil*. **Suppl., 66**, 51–

66. https://doi.org/10.1530/biosciprocs.18.0004
54. Chelucci S, Pasciu V, Succu S, Addis D, Leoni GG, Manca ME, Naitana S & Berlinguer F (2015) *Theriogenology* **14**, 1–12.
55. Insani K, Sri R, Agung P & Aries S (2014) *J Biotropika*, **2(3)**, 142-147 .
56. Agarwal A, Nallella KP, Allamaneni SSR & Said TM (2004) *Reprod. Biomed. Online* **8(6)**,616–627. https://doi.org/10.1016/s1472-6483(10)61641-0
57. Kumar P, Pawaria S, Dalal J, Ravesh S, Bharadwaj S, Jerome A, Kumar D, Jan MH & Yadav PS (2019) *Anim. Reprod. Sci.* **209**, 106166. https://doi.org/10.1016/j.anireprosci.2019.106166
58. Hayati A, Mangkoewidjojo S, Hinting A & Moeljopawiro S (2006) *J. Biol. Res.* **11**, 151–154.
59. Desai NR, Mahfouz R, Sharma R, Gupta S & Agarwal A (2010) *Fertil. Steril*. **94(4)**, 1541–1543. https://doi.org/10.1016/j.fertnstert.2009.12.041
60. Liu Y & O'Flaherty C (2017) *Asian J Andrology* **19(1)**, 73-79.
61. Morelli T & O'Flaherty C (2015) *Reproduction* **149(1)**, 113-123.
62. Toborek M & Hennnig B (1994) *American J Clinical Nutrition* **59(1),** 60-65.
63. Cha SJ, Kim H, Choi H-J Lee S & Kim K (2017) *Oxidative Medicine and Cellular Longevity* **2017**, 2818565.
64. Yeo W-S, Lee S-J, Lee J-R & Kim K-P (2008) *BMB Reports* **41(3**), 194-203.
65. Batthyány C, Bartesaghi S, Mastrogiovanni M, Lima A, Demicheli V & Radi R (2017) *Antioxidants and Redox Signalling* **26(7)**, ars2016. 6787.
66. O'Flaherty C & Matsushita-Fournier D (2017) *Biology of Reproduction* **97(4)**, 577-585.
67. Andreea A & Stela Z (2010) *Rom. Biotechnol. Lett*., **15(3),** 33–41.
68. Khan IM, Xu D, Cao Z, Liu H, Khan A, Rahman SU, Ahmed JZ, Raheem MA & Zhang Y (2021) *Pak. J. Zool.* **53(4)**, 1–11. https://doi.org/10.17582/journal.pjz/20191006091046
69. Alvarez JG & Storey BT (1982) *Biol. Reprod.*, **27(5)**:1102–1108. https://doi.org/10.1095/biolreprod27.5.1102
70. Gadea J, Gumbo D, Novass C, Zquezf AZ, Grullol A & Gardo GC (2007) *Andrology*, **7**:1–10.
71. Rizkallah N, Chambers CG, de Graaf SPD, & Rickard JP (2022) *Animals*, **12(3)**:1–18. https://doi.org/10.3390/ani12030244
72. Dalal J, Kumar P, Chandolia RK, Pawaria S, Rajendran R, Sheoran S, Andonissamy J., & Kumar D (2022) *Sci. Rep.*, **12(1)**:9015. https://doi.org/10.1038/s41598-022-12465-1
73. Mazzilli F, Rossi T, Sabatini L, Pulcinelli FM, Rapone S, Dondero F & Gazzaniga PP (1995) *Acta Eur. Fertil*., **26(4)**:145–148.
74. Yeste M (2016) *Theriogenology* **85(1)**:47–64. https://doi.org/10.1016/j.theriogenology.2015.09.047
75. Abouelezz MF, Montaser AE, Hussein MS, Eldesouky AM, Badr MR, Hegab AO, Balboulaa AZ & Zaabela SM (2016) *Reprod. Biol*., **214**:1–9.
76. Soltan WM, El-Siefy EME & Gabr SA (2023) *Egypt. J. Nutr. Feeds* **26(1)**, 27–34. https://doi.org/10.21608/ejnf.2023.297881
77. Patel HA, Siddiquee GM, Chaudhari DV, and Suthar VS (2016) *Vet. World* **9(3)**, 299–303. https://doi.org/10.14202/vetworld.2016.299-303
78. Varghese O, Dhami AJ, Hadiya KK, Patel J. A & Parmar SC (2015) *Indian J. Anim. Reprod*. **36(2)**, 39-45.
79. Eliraqy EZ, Shafik BM, Hussein YS, Alsenosy AA, Abdallah EA & Saad MF (2022) *Adv. Anim. Vet. Sci*. **10(10)**, 2133–2141. https://doi.org/10.1016/0014-4827(72)90303-5
80. Bassuony NI, Assi MM, Sakr AA, Bakr AA, Anwar DA & Badr MR (2023) *Alexandria J. Vet. Sci*. **76(1)**, 108–116. https://doi.org/10.5455/ajvs.124422
81. Khalek AEA, Nagar HAE & Ibrahim OM (2016) *J. Agric. Econ. Soc. Sci., Mansoura. Univ.*, **7(7)**, 225–231.
82. Ramazani N, Mahd Gharebagh F, Soleimanzadeh A, Arslan HO, Keles E, Gradinarska-Yanakieva DG, Arslan-Acaröz D, Zhandi M, Baran A, Ayen E & Dinç DA (2023) *Vet. Med. Sci.* **9(4),** 1791–1802. https://doi.org/10.1002/vms3.1158
83. Ahmed H, Jahan S, Salman MM & Ullah F (2019) *Theriogenology* **134**, 18–23. https://doi.org/10.1016/j.theriogenology.2019.05.012
84. El-Khawagah ARM, Kandiel MMM & Samir H (2020) *Front. Vet. Sci*., **7,** 604460. https://doi.org/10.3389/fvets.2020.604460
85. Kumar R, Singh VK, Chhillar S & Atreja S. K (2013) *Reprod. Dom. Anim.*, **48(3)**, 407–415. https://doi.org/10.1111/rda.12088

86. Kumar N, Gaur M, Jhamb D & Vyas J (2023) *Indian J. Vet. Sci. Biotech.*, **19(3),** 70.
87. Ercal N, Gurer-Orhan H & Aykin-Burns N (2001) *Curr. Top. Med. Chem*. **1(6)**, 529–539. https://doi.org/10.2174/1568026013394831
88. Khan J, Tahir MZ, Khalid A, Sattar A & Ahmad N (2017) *Reprod. Domest. Anim.* **52(2),** 265–268. https://doi.org/10.1111/rda.12893
89. Khumran AM, Yimer N, Rosnina Y, Ariff MO, Wahid H, Kaka A, Ebrahimi M, & Sarsaifi K (2015) *Anim. Reprod. Sci.* **163,** 128–134. https://doi.org/10.1016/j.anireprosci.2015.10.007
90. Meister A & Anderson ME (1983) *Annu. Rev. Biochem.* **52,** 711–760. https://doi.org/10.1146/annurev.bi.52.070183.003431
91. Kaeoket K, Chanapiwat P, Tummaruk P, & Techakumphu M (2010) *Asian. J. Androl*. **12(5),** 760–765. https://doi.org/10.1038/aja.2010.48
92. Kulaksiz R & Daskin A (2010) *Ank. Üniv. Vet. Fak. Derg*. **7,** 151–156.
93. Sariözkan S, Bucak MN, Tuncer PB, Ulutaş PA & Bilgen A (2009) *Cryobiology* **58(2),** 134–138. https://doi.org/10.1016/j.cryobiol.2008.11.006
94. Memon AA, Wahid H, Rosnina Y, Gohb YM, Ebrahimi M, Nadiac FM & Audrey G (2011) *Elixir Int. J.* **38**, 4100–4104.
95. Bucak MN, Ateşşahin A, Varişli O, Yüce A, Tekin N & Akçay A (2006) *Theriogenology* **67(5),** 1060–1067. https://doi.org/10.1016/j.theriogenology.2006.12.004
96. Kek SP, Chin NL, Yusof YA, Tan SW & Chua LS (2014) *Agric, Agric. Sci. Procedia*. **2,** 150–155. https://doi.org/10.1016/j.aaspro.2014.11.022
97. Fuller BJ (2004) *CryoLetters* **25(6),** 375–388.
98. Chung ELT, Nayan N, Nasir NSM, Hing PSA, Ramli S, Rahman MHA & Kamalludin MH (2019) *Anim. J. Health. Prod*. **7(4),** 171–178.
99. Chaudhary KF, Suthar BN, Nakhashi HC & Mohapatra SK (2022) *Rumin. Sci*., **11(1),** 57–62.
100. Tvrda E, Greifová H, Mackovich A, Hashim F & Lukáč N (2018) *Czech J. Anim. Sci.* **63(7)**, 247–255. https://doi.org/10.17221/33/2017-CJAS
101. Navjot K, Manjinder S, Lonare MK & Digvijay S (2020) *Rumin. Sci.* **9(2)**, 215–222.
102. Sheshtawy RIE, Sisy GAE & Nattat WSE (2016) *Asian. Pac. J. Reprod*. **5(4)**, 35–339.
103. Halvorsen BL, Holte K, Myhrstad MC, Barikmo I, Hvattum E, Remberg SF, Wold, AB, Haffner K, Baugerød H, Andersen LF, Moskaug Ø, Jacobs DR Jr. & Blomhoff R (2002) *J. Nutr.* **132(3),** 461–471. https://doi.org/10.1093/jn/132.3.461
104. Giampieri F, Alvarez-Suarez JM, Mazzoni L, Forbes-Hernandez TY, Gasparrini M, Gonzàlez-Paramàs AM, Santos-Buelga C, Quiles JL, Bompadre S, Mezzetti B & Battino M (2014) *Molecules* **19(6)**, 7798–7816. https://doi.org/10.3390/molecules19067798
105. Brito A, Ramirez JE, Areche C, Sepúlveda B & Simirgiotis MJ (2014) *Molecules* **19(11)**, 17400–17421. https://doi.org/10.3390/molecules191117400
106. Park SH & Yu IJ (2016) *J. Anim. Reprod. Biotech*. **30(1)**, 1–6. https://doi.org/10.12750/JET.2015.30.1.1
107. Hussein YS (2018) *Egypt. J. Anim. Prod.* **55(1)**, 1–6.
108. Ali MM, Banana HJ & Ibrahim FF (2021) *Indian J Phys Anthropol Human Genetics* 48, 81-86.
109. Singh P, Agarwal S, Singh H, Singh S, Verma PK, Butt MS & Sharma U (2020) *Int. J. Curr. Microbiol. Appl Sci.* **9(7)**, 3089–3099. https://doi.org/10.20546/ijcmas.2020.907.364
110. Singh MM & Sharma A (2018) *Indian J. Anim. Sci*. **88(7),** 791–794. https://doi.org/10.56093/ijans.v88i7.81425
111. Prihantoko KD, Yuliastuti F, Haniarti H, Kusumawati A, Widayati DT & Budiyanto A (2020) *Earth and Environmental Science Conference Proceedings* **478**, doi: 10.1088/1755-1315/478/1/012042.
112. Chuawongboon P, Sirisathien S, Pongpeng J, Sakhong D, Nagai T & Vongpralub T (2017) *Anim. Sci. J*. **88(9)**, 1311–1320. https://doi.org/10.1111/asj.12798
113. Pytlík J, Codl R, Ducháček J, Georgijevič Savvulidi FGG, Vrhel M & Stádník L (2023) *Czech J. Anim. Sci.*, **68(2),** 64–71. https://doi.org/10.17221/223/2022-CJAS
114. Avdatek F, Yeni D, İnanç ME, Çil B, Tuncer BP, Türkmen R & Taşdemir U

(2018) *Andrologia* **2018**, 12975. https://doi.org/10.1111/and.12975
115. Korkmaz F, Malama E, Siuda M, Leiding C & Bollwein H (2017) *Anim. Reprod. Sci*. **185(1)**, 18–27. https://doi.org/10.1016/j.anireprosci.2017.07.017
116. Chikhaliya PS, Ahlawat AR, Singh VK, Vijyeta HP, Odedra MD & Vala KB (2018) *Int. J. Curr. Microbiol. Appl. Sci.* **7(7),** 1441–1447. https://doi.org/10.20546/ijcmas.2018.707.171
117. Eidan SM (2016) *Anim. Rep. Sci*. **167,** 1–7. https://doi.org/10.1016/j.anireprosci.2016.01.014
118. Khan H, Khan M, Qureshi MS, Ahmad S, Gohar A, Ullah H, Ullah F, Hussain A, Khatri P, Shah SSA, Rehman H & Khan A (2017) *Pak. J. Zool*. **49(4),** 1243–1249. https://doi.org/10.17582/journal.pjz/2017.49.4.1243.1249
119. Park SH & Yu IJ (2017) *J. Anim. Reprod. Biotech.* **32(1)**, 9–15. https://doi.org/10.12750/JET.2017.32.1.9
120. Tvrda E, Mackovich A, Greifova H, Hashim F & Lukac N (2017) *Vet. Med.* **62(8),** 429–436. https://doi.org/10.17221/86/2017-VETMED
121. Martinez-Soto JC, de DiosHourcade J, Gutiérrez-Adán A, Landeras JL & Gadea J (2010) *Asian J. Androl.* **12(3),** 431–441. https://doi.org/10.1038/aja.2009.92
122. Xiao Y, Wu Z & Wang M (2018) *Zygote* **26(3)**, 220–223. https://doi.org/10.1017/S0967199418000126
123. Anane R & Creppy EE (2001) *Hum Exp Toxicol.* **20(9),** 477–481. https://doi.org/10.1191/096032701682693053
124. Yousef MI, Abdallah GA & Kamel KI (2003) *Anim. Reprod. Sci.* **76(1–2)**, 99–111. https://doi.org/10.1016/s0378-4320(02)00226-9.
125. Simonik O, Rajmon R, Stádník L, Šichtař J, Beran J, Ducháček J, Hodek P & Trefil P (2016) *Czech J. Anim. Sci.* **61(12)**, 560–567. https://doi.org/10.17221/27/2016-CJAS
126. Hu JH, Jiang ZL, Lv RK, Li QW, Zhang SS, Zan LS, Li YK & Li X (2011) *Cryobiology* **62(1)**, 83–87. https://doi.org/10.1016/j.cryobiol.2010.12.007
127. Beran J, Šimoník O, Stádník L, Rajmon R, Ducháček J, Krejcárková A, Doležalová M., & Šichtař J (2013) *Acta Univ. Agric. et Silvic. Mendelianae Brun*. **61(6)**, 1575–1581. https://doi.org/10.11118/actaun201361061575
128. Stadnik L, Rajmon R., Beran J, Šimoník O, Doležalová M, Šichtař J, Stupka R & Folková P (2015) *Acta Vet. Brno*, **84(2),** 125–131. https://doi.org/10.2754/avb201584020125
129. Jorsaraei SGA, Shibahara H, Ayustawait HY, Shiraishi Y, Khalatbari A, Pasha YY, & Suzuki M (2008) *Iran. J. Reprod. Med.*, **6(3),** 157–165.
130. Khaki A, Batavani RA & Najafi G (2013) *Vet. Res. Forum.* **4(1)**, 7–12.
131. Maalik A, Khan F, Mumtaz A, Mehmood A, Azhar S, Atif M, Karim S, Altaf Y & Tariq I (2014) *Trop. J. Pharm. Res.* **13(9)**, 1561–1566. https://doi.org/10.4314/tjpr.v13i9.26
132. Omur AD & Uluyol O (2022) *J. Clin. Vet. Res*. **2(1)**, 1–6.
133. Marcinkiewicz J & Kontny E (2014) *Amino Acids* **46(1)**, 7–20. https://doi.org/10.1007/s00726-012-1361-4
134. Bouckenooghe T, Remacle C & Reusens B (2006) *Curr. Opin. Clin. Nutr. Metab. Care*. **9(6),** 728–733. https://doi.org/10.1097/01.mco.0000247469.26414.55
135. Nadhem AHA (2019) *J. Appl. Vet. Sci*. **4(2),** 71–79.
136. Foote RH (2002) *J. Anim. Sci*. **80(2),** 1–10. https://doi.org/10.2527/animalsci2002.80E-Suppl_21a
137. Ali MM & Husam JHB (2020) *Plant Arch.* **20(1)**, 1209–1216.
138. Perez L, Arias ME, Sanchez R & Felmer R (2015) *Andrologia* **20**, 1–6.