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Role of semen additives in cryopreservation

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Abstract
Sperm cryopreservation is an effective method for the management and preservation of male fertility in domestic animals. Cryopreservation has been started since a long time and plays a crucial role in semen preservation. Sperm membrane has high amount of polyunsaturated fatty acids, the changes occuring during the freezing process causes lipid peroxidation and oxidative stress. Additives are being used to reduce the stress and improve the semen quality. Our ultimate goal is to increase milk production and the number of calves in an animal's reproductive life. All the breedable females can be covered for insemination by increasing the production of semen and improving the quality. This article deals with different types of additives and studies on some important additives.

Keywords: Antioxidants, cryopreservation, semen additives, spermatozoa

Introduction
Artificial insemination is the 1st generation and easiest method of increasing productivity. Artificial insemination is an emerging technology in the field of reproduction. It played a critical role in improving the genetic progress of bovines (Bishist *et al.*, 2020) [5]. Because of artificial insemination, the superior quality male germplasm from good bulls is exploited to a very great extent, and a large number of buffaloes are inseminated with a single ejaculate (Hussain *et al.*, 2018) [16]. It helps to prevent the spreading of venereal diseases. Semen cryopreservation is an essential technique for the long-term storage of semen. Artificial insemination is only possible because of semen cryopreservation. However, the role of semen cryopreservation is minimal in the case of buffalo because of the low keeping quality and poor freezability of semen (Bishist *et al.*, 2020) [5]. In buffalo, artificial insemination has been practiced for a long time, but the conception rate in buffalo is lower (30%) than in cattle (Barile, 2012) [4]. Because of the lipid content variation, a buffalo spermatozoa is very prone to cryopreservation's deleterious effects. The sperm plasma membrane is very rich in polyunsaturated fatty acids. So it is very vulnerable to lipid peroxidation. The sperm plasma membrane is affected due to the freezing-thawing process. Cholesterol to phospholipid ratio is less in buffalo in comparison to other species which poses a great risk of damage to the plasma membrane of spermatozoa.

Deleterious effects of cryopreservation
The first time semen cryopreservation was attempted by Lazaro Spallanzani (1776) as he tried to cryopreserve the spermatozoa by cooling with help of snow (Royere *et al.*, 1996) [35]. Later, a turning point came when the cryoprotective properties of glycerol were discovered (Polge *et al.*, 1949) [34]. Since the time of discovery of glycerol, cryopreservation started at commercial levels. Cryopreservation poses risk of damage to various structures of spermatozoa which can hamper the functioning of sperm cell. Cryopreservation also causes a reduction in temperature, osmotic stress, pH fluctuations, ice crystal formation, oxidative stress, cold shock, lipid scrambling and peroxidation (Hezavehei *et al.*, 2018) [14]. During the time of cryopreservation large ice crystal formation takes place. In the absence of cryoprotective agents, cold shock and the induction of ice crystal formation can lead to the destruction of organelles in the sperm cells (Abdel Hafez *et al.*, 2009) [1]. Cryotolerance is a function of amount of lipid present in the plasma membrane of spermatozoa. Different species sperm has different level of cryotolerance due to presence of fatty acid profile and omega-3/ omega-6 ratio in spermatozoa in the plamsa membrane (Esmaeili *et al.*, 2015) [12]. Plasma membrane is the most critical part of sperm cell to be affected by cryopreservation process. During the freezing process, cholesterol efflux takes place from the sperm plasma membrane which leads to reduction in cholesterol to

phospholipid ratio. Lipid peroxidation takes place and reactive oxygen species like hydrogen peroxide, hydroxyl radical, superoxide radicals, peroxyl, nitroxyl, peroxynitroxyl, nitrous oxide etc. produce. Out of all the ROS, hydrogen peroxide and hydroxyl radicals are most harmful to spermatozoa (Agarwal *et al.*, 2014) [2]. In addition to this, osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants, intracellular and extracellular ice crystals affect the sperm to a great extent (Medeiros *et al.*, 2002) [25]. Reactive oxygen species are necessary for various processes like capacitaion, acrosomal reaction and hyperactivation. But when the amount of ROS overboards the amount of natural antioxidants present in seminal plasma, it leads to oxidative stress posing damages to various organelles of spermatozoa like reorganization of phospholipids, disruption of disulphide bonds between proteins, DNA damage, protein damage, mitochondrial damage. All these factors reduce the sperm vaibility and fertility. Oxidative stress also affects the embryo quality and blastocyst formation rate (De Castro *et al.*, 2016) [9]. Interestingly, it has been observed that the ultrastructural integrity can be reduced by rapid freezing and preserved the integrity of sperm heads and plasma membrane compared with slow freezing (Serafini *et al.*, 1986) [36]. Now days, semen additives are also being studied to see the effect on cryopreservation process.

Semen additives

Semen additives are the substances which are added at the processing time and help in improvement of semen quality by reducing the oxidative stress either by quenching the ROS or maintaining the plasma membrane stability. There are various kinds of additives: antioxidants, antioxidant preservatives, amino acids, methylxanthines, sugar/plysaccharides, trace elements and plant extracts.

Table 1: Classification of antioxidants

Classes	Examples
Antioxidants	Vitamin-E, Ascorbic acid, Superoxide desmutase (SOD) and Glutathione peroxidase (GPX), Resveratrol, Mitoquinone
Antioxidant Preservatives	Butylated hydroxy toluene (BHT), Butylated hydroxy anisole
Methylxanthine	Pentoxifylline (PTX) and Caffeine
Trace Elements	Copper, Zinc, Selenium
Amino acids/Proteins	Cysteine, Taurine, Hypotaurine
Sugar/Polysaccharides	Trehalose, Hyaluronic acid, cholesterol loaded cyclodextrins

Antioxidants and antioxidants preservatives

Antioxidants are the agents, which break the oxidative chain reaction -eliminating, taking up, or reducing the formation of ROS (Bansal and Bilaspuri, 2011) [3] and thereby reduce the oxidative stress (Miller *et al.*, 1993; Kumar and Mahmood, 2001) [27, 18]. Antioxidants are of two types; enzymatic antioxidants and non-enzymatic antioxidants. Another classification is preventive antioxidants (Mitoquinone, lactoferrin) and scavenging antioxidants (Vitamin E, vitamin C, superoxide dismutase, catalase, glutathione peroxidase). Preventing antioxidants are those which prevent the formation of reactive oxygen species and scavenging atioxidants are those which quench the already formed ROS. GSH/glutathione peroxidase mainly acts as scavenging antioxidants in the epididymis and testes (Mora-Esteves and Shin, 2013) [28], and helps in preserving sperm viability and motility by confering protection on the lipid constituents of the sperm membrane (Lanzafame *et al.*, 2009) [19]. Butylated hydroxytoluene is a perfect example of antioxidant preservatives. BHT is an organic soluble molecule used to prevent damage to the sperm plasma membrane and the phospholipid bilayer (Hammerstedt *et al.*, 1978) [13]. It protects the spermatozoa from cold shock by reduction in lipid peroxidation and oxidative stress. BHT acts as an antioxidant as it scavenges the reactive oxygen species, targeting spermatozoa (Killian *et al.*, 1989) [17]. It also acts as an antiviral and antimicrobial agent.

Methylxanthine

Methylxanthines are phosphodiesterase inhibitors. The cAMP phosphodiesterase enzyme causes the breakdown of cAMP, and lower down its concentration. As it inhibits cAMP phosphodiesterase enzyme, causes a raise in the level of cAMP and increases the rate of glycolysis. Hence, spermatozoal motility increased due to production of more number of ATPs (Hicks *et al.*, 1972) [15]. Examples of methylxanthines are caffeine and pentoxiphyllines.

Amino acids

Amino acids have an important role in protection of spermatozoa against oxidative stress caused by various changes during cryopreservation process. Various examples of amino acids which protect the sperm from damages are cysteine, taurine, hypotaurine. Cysteine is a sulphur containing amino acid with thiol group. As it is an important component of nucleic acid present in head of sperm helps in formation of DNA and also maintains the integrity of the DNA. This is also a precursor of natural glutathione and protects the spermatozoa through its indirect effect on ROS through production of glutathione (Memon *et al.*, 2011) [26]. Taurine is a sulfonic amino acid as it is a derivative of methane sulphonic acid. Being a non-enzymatic antioxidant, it decreases the cellular damage caused by the process of cryopreservation. It acts as a non- permeating cryoprotectant**.** Other amino acids like hypotaurine also scavenge reactive oxygen species and improve semen quality.

Sugars/Polysaccharides

Trehalose is a nonreducing disaccharide sugar. It reduces intracellular ice crystal formation during cryopreservation when used in higher concentration (Büyükleblebici *et al*, 2014) [6]. However, when it is used in low concentration it displays the antioxidative properties and improves by reducing LPO (Tuncer *et al.*, 2013) [39]. It forms specific interactions with the membrane phospholipids to protect the membrane from damage and help stabilize the sperm plasma membrane. It is a membrane stabilizer and also acts as a non-permeating cryoprotectant. Cyclodextrins are the products of the degradation of starch with the help of enzymes. They possess a unique quality to remove cholesterol from the cell membrane when used alone. However, when cyclodextrins are loaded with cholesterol, they can introduce cholesterols in the sperm cell membrane (Lone *et al.*, 2016) [21]. Cyclodextrins has an internal hydrophobic core and outer hydrophilic core and high affinity to sterols (Navratil *et al.*, 2003) [31], due to which it can incorporate cholesterol in the cell membrane (Christian *et al.*, 1997) [8]. A decrease in C: P ratio can cause cryocapacitation and programmed cell death

(Martin *et al.*, 2004; Martin *et al.*, 2007) [24, 23]. CLC maintains the C:P ratio of sperm plasma membrane and protects it from cryocapacitation like changes.

Table 2: List of some important semen additives and their effects

Semen Additives	MOA	Effect	References
Vit E (5 IU/ml)- Ram semen	Best antioxidant, Scavenger of ROS	MDA concentration (18.78±4.5 µM/ml vs 43.68±3.5 µM/ml in control) Superoxide dismutase 89.5±4.6 IU/ml plasma vs 32.8±5.6 IU/ml plasma in control Glutathione peroxidase content was also higher 137.9±7.4 nM/ml plasma than control 22.1±3.2 nM/ml plasma	Zeitoun *et al.* (2014) [40]
Ascorbic acid (2.5 mM) + GSH (2 mM) - Holstein bulls	Antioxidants, scavenger of ROS	Increase in sperm motility and viability than control. MDA concentration decreased.	Eidan, 2016 [11]
Superoxide dismutase and Reduced glutathione (50 IU/ml+ 0.5 mM, Bull semen	Antioxidant function, scavenger of ROS	Motility 49% Live sperm 69% HOST 51% Acrosomal integrity 70%- in combination SOD+GSH (50 IU/ml+ 0.5mM higher than other treatments and control.	Murtaza *et al.* (2018) [29]
Melatonin (2 mM)- Hariana bull semen	Hydroxyl radical scavenger, neutralizes ROS and nitrogen species	Motility% (56 vs 47%) Viability% (61 vs 50%) Sperm abnormality% (7 vs 10%) Acrosomal integrity% (75 vs 55%) HOST% (66 vs 56%) Melatonin vs control	Perumal *et al.* (2013) [33]
Mitoquinone (200 nM) - Murrah buffalo bull	Mitochondria targeted antioxidant, Lipophillic, enter into mitochondria and prevent the generation of ROS	Progressive motility% (45 vs 35%), Live sperm % (54 vs 45%) and plasma membrane intact sperm % (51 vs 43%) were higher in comparison to control.	Tiwari *et al.* (2022) [38]
Resveratrol (50 µM) - Goat semen	Antioxidant, anticancer, anti-aging, anti-inflammatory, cardioprotective and neuroprotective actions, scavenger of superoxide and peroxynitrite radicals	10 or 50 µM concentration leads to an increase in total motility, progressive motility, membrane and acrosome integrity, mitochondrial activity, percentage of viable spermatozoa in comparison with control.	Lv *et al.* (2019) [22]
Butylated hydroxytoluene (BHT)-0.5-1 mM Murrah Buffalo	BHT is a lipid-soluble antioxidant and synthetic analogue of vitamin E, increases intra-cytoplasmic fluidity	Post thaw semen parameters PM% (69 vs 63%) Liveability % (78 vs 74%) HOST % (71 vs 64%) Acrosomal integrity%(84 vs 82%) Higher in BHT in comparison to control.	Nain *et al.* (2023) [30]
Pentoxiphylline (PTX) -3.6 mM Buffalo Bull	Phosphodiestrase inhibitor, Increase cAMP level and motility of spermatozoa	Significantly higher sperm motility was observed in PTX than theophylline and theobromine. All the sperm parameters were significantly higher at post-thaw stage in PTX.	Bishist *et al.* (2020) [5]
Cysteine Hydrochloride (1 mM)- Crossbreed Jersey bulls	Precursor of natural glutathione, part of nucleic acid	High post-thaw motility, MMP, decreased lipid peroxidation, all the sperm kinetic parameters like VAP, VSL, and ALH got increased	Perumal *et al.* (2011) [32]
Taurine (50 mM) and trehalose (100mM)- Karan Fries bull semen	non- permeating cryoprotectant, anti-oxidant	Capacitated spermatozoa % (18 vs 20, 24 %), Lipid peroxidation (nmol MDA/10^8 cells) - 1.49 vs 1.51, 2.61) higher in taurine in comparison to trehalose and control	Chillar *et al.* (2012) [7]
Trehalose 100 mM- Kankrej bull semen	**Non-permeating cryoprotectant, membrane stabilizer**	Mean AST and ALT activities were 63.24±1.06 U/L and 22.97±0.71 U/L, respectively at the post-thaw stage	Thumar (2017) [37]
Anandamide (1 µM in 20,15,10 and 5 million/straw) - Murrah buffalo	Lipogenic effect, Inhibit O2 consumption and ATP production which preserve energy and acquisition of fertilising potential	Live spermatozoa % (56,55,52,48 vs 48,46,41,35) LAI % (50.49.46,43 vs 44, 43, 38, 31) in 20,15,10 and 5 million doses respectively. Capacitated spermatozoa and sperm motility significantly reduced in 5 million sperm doses.	Lone *et al.* (2020) [20]
Cholesterol loaded cyclodextrin (1 mg/120 million sperm)- Buffalo	Inserts cholesterol in plasma membrane, reduces cryocapacitation	Progressive motility (82 vs 77%) Liveability (85 vs 81%) Acrosome integrity (85 vs 80%) HOS response (75 vs 70%) LPO (282 vs 298%) ROS (105 vs 119%) -CLC vs control	Lone *et al.* (2016) [21]

Conclusion

Sperm cryopreservation is a crucial technique for artificial insemination of animals. Various additives are being studied to increase the quality of semen. Combination of additives is also being tried now a day with good results. Hence, the stresses caused by cryopreservation can be minimised by using additives and functional properties of semen can be improved. Also understanding the molecular modification caused by additives can also be helpful in optimization of semen quality.

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