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Additives used in semen preservation in animals: A short review

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Abstract
Mammalian spermatozoa are extremely sensitive to oxidative damage wherein lipid peroxidation plays the most significant role in aging of the spermatozoa along with shortening its life-span in vivo and thereby affecting the preservation of semen for artificial insemination. In this developing world of artificial breeding in animals, the superior quality male germplasm from elite male is exploited to maximum possible extent by artificially inseminating large number of female animals merely from a single ejaculate. For this purpose, a good quality semen extender is required which plays an indispensable role in improving the post thaw semen quality in terms of sperm viability, motility, plasma membrane and acrosomal integrity (PMAI), mitochondrial membrane potential (MMP), sperm kinematics etc. A number of semen additives are incorporated during extension of semen before preservation which imparts anti-oxidant and sperm membrane stabilization properties to improve post-thaw semen quality. Thus it is important to disseminate the knowledge about the use of various types of semen extenders and additives in animal semen preservation to improve the extent of consuming superior male germplasm in veterinary science and animal husbandry.

Keywords: additives, animals, preservation, sperm

Introduction
Sperm cells, being the endpoint of male spermatogenesis, have particular anatomic and metabolic features, and their cryopreservation and storage currently require liquid nitrogen or ultralow refrigeration methods for long or short term storage. Conserving sperms requires routine maintenance and extensive space requirements, and such conservations have several purposes such as artificial reproductive technologies (ART), species conservation and clinical medicine. The combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control are the key factors that affect the life-span of spermatozoa. The major disadvantage of cryopreservation is death of the half of the sperm population during freezing and thawing procedure even if most adoptive preservation technique is followed. In addition to this, changes in temperature impart changes on the composition and structure of sperm plasma membrane integrity by adversely modifying their function (Krogenaes *et al.*, 1994) [47] submission to osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment (Medeiros *et al.*, 2002) [55]. The damaging effects of cooling and freezing upon sperm membrane varies among domestic species and is influenced by several elements namely cholesterol/ phospholipids ratio, content of lipids in the bilayer, degree of hydrocarbon chain saturation and protein/ phospholipid ratio (Medeiros *et al.*, 2002) [55]. Boar sperm is the most sensitive; bull, ram and stallion are very sensitive; dog and cat are somewhat sensitive; rabbit, human, and rooster are less sensitive to cold shock (Parks, J. E., 1997) [63].

Role of extenders in cryopreservation of semen
Different factors, including osmotic pressure, physico-chemical stresses and freeze-thaw temperature variations affects the semen quality parameters such as viability, motility and membrane integrity (Alvarez and Storey, 1992) [8]. Mammalian spermatozoa are extremely sensitive to oxidative damage, and the different stressors generates Reactive Oxygen Species.

(ROS) and lipid peroxidation of the cell membrane, which will ultimately affects the spermatozoa (Wang *et al.,* 1997) [94]. At present, it is generally accepted that the consequences of sperm cryo-injury caused by the cryopreservation procedure are impaired transport and poor survival in the female reproductive tract (Salamon and Maxwell, 1995) [78]. Although bovine semen has a natural defense system against the oxidative stress, it is considered insufficient under cryopreservation-mediated stress, and so reinforcement of semen extender with suitable additives is suggested to reduce oxidative damage during freeze-thawing of bull and buffalo spermatozoa (Ansari *et al.,* 2011a) [12]. Equine sperm are particularly known to be susceptible to oxidative stress, relative to other species, because of their high content of unsaturated fatty acids.

Lipid peroxidation plays a leading role in aging of spermatozoa, shortening its life-span in vivo and affecting the preservation of semen for artificial insemination (Alvarez and Storey, 1982) [10]. The process of peroxidation induces structural alterations; particularly in the acrosomal region of the sperm cell, a fast and irreversible loss of motility, a deep change in metabolism and a high rate of release of intracellular components. Lipid peroxidation has been defined as an important aspect of oxidative stress in mammalian spermatozoa for many years (Jones and Mann, 1973; Jones *et al.,* 1979) [44, 45]. The susceptibility of spermatozoa to oxidative damage is attributed to the high concentration of unsaturated fatty acids in membrane phospholipids, and limited antioxidant capacity of spermatozoa as well as the ability of spermatozoa to generate reactive oxygen species (ROS) (Aitken, 1995; Storey, 1997) [5, 87]. Spermatozoa are capable of generating ROS (Aitken *et al.,* 1997; Balla *et al.,* 2001) [4, 14], and the controlled generation of ROS may have physiological functions in signaling events controlling sperm capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion. The uncontrolled production of ROS by defective spermatozoa can have a detrimental effect on sperm function (Baumber *et al.,* 2000) [20]. Oxidative stress appears as a consequence of this extreme ROS production or from alteration in the antioxidative mechanisms of defense.

Extenders have a vital role in the preservation of sperm cell and its quality parameters such as viability, motility, acrosome and membrane integrity etc. Semen extenders contain buffering system to maintain pH of the medium (Tris, sodium phosphate, citric acid), cryoshock preservatives (glycerin, egg yolk, soy-lecithin, milk), one or more sugars to provide energy (glucose, lactose, raffinose, fructose, saccharose, or trehalose), salts (sodium citrate, citric acid) and guarantee the microbial free environment i.e. antibiotics (streptomycin, penicillin, polymixin B) (Evans and Maxwell, 1987) [32]. The fortification of extender by various semen additives improves motility as well as fertility of spermatozoa.

A number of additives have been studied over the past few years and their effect on improving the quality of various parameters of the semen has been reported. These additives include antioxidants, sugars, cholesterol and proteins, etc., out of which, few are effective and has been reported to improve the semen quality even in post thaw stage. Such additives not only improve *in vitro* fertility, but also improves the in vivo fertility in all species of animals. Whether the semen is to be used immediately or preserved, it should always be mixed with an appropriate extender within a few minutes after collection to maximize sperm longevity. An initial semen/extender dilution ratio of 1:1 to 1:2 is generally adequate if semen is not to be stored for more than 1 to 2 hours before insemination. A warmed extender can also be placed in the semen receptacle before collection so that the sperm comes in contact with this supportive, and this procedure can be beneficial for animals whose seminal plasma seems to depress longevity of sperm motility or otherwise interfere with fertility (Brinsko and Hartman, 2011) [24]. Further, properly formulated semen extenders improves sperm survival during the interval between collection and insemination. Such semen extenders contain antimicrobials primarily to limit or prevent bacterial growth while the semen is being stored above 4° to 6° C, rather than to eliminate pathogens. Also such antimicrobials may also limit the establishment of endometritis in susceptible females in which A.I is done.

Different types of additives and their function

Lactose, sucrose, raffinose, trehalose and dextrans are not able to diffuse across the plasma membrane, creating an osmotic pressure that induces cell dehydration and a lower incidence of intracellular ice formation. These sugars interact with phospholipids in the plasma membrane, increasing sperm survival to cryopreservation (Aisen *et al.,* 2002) [3]. The addition of high concentrations of trehalose to sperm extender provides the best protection with regard to post-thaw motility parameters, recovery rates, thermal resistance, and acrosome integrity (Aboagla and Terada, 2003) [1]. This disaccharide increases membrane fluidity before freezing, leading to greater resistance of spermatozoa against freeze-thawing damage (Aboagla and Terada, 2003) [1].

Antioxidants

Antioxidants are the agents, which break the oxidative chain reaction -eliminating, taking up, or reducing the formation of ROS (Bansal and Bilaspuri, 2011) [18] and thereby reduces the oxidative stress (Miller *et al.,* 1993; Kumar and Mahmood, 2001) [57, 49]. The antioxidants check the chemical breakdown of the substrate resulting from oxidation and neutralizes the free radicals thus reducing the risk of damage to spermatozoa during cryopreservation (Pe˜na et al, 2003; Roca et al, 2004; Strzezek J., 2002) [66, 76, 88]. Antioxidants may be preventive antioxidants (metal chelators or binding proteins, such as lactoferrin and transferring), which prevent the formation of ROS, or scavenging antioxidants, like vitamins C and E, which removes the ROS that is already present (Lampiao, F. 2012) [50]. Based on their chemical property, antioxidants may also be categorized as enzymatic (e.g. glutathione reductase or, GSH, SOD, and catalase), and non-enzymatic (e.g. vitamins C, E, and B; carotenoids; carnitines; cysteines, pentoxifylline, metals, taurine, hypotaurine, and albumin) (Bansal and Bilaspuri, 2011) [18]. The non-enzymatic oxidants are acquired from fruits or vegetables containing the supplements (Lampiao, F. 2012) [50]. Antioxidants also act as motility enhancing agents, and thus improves the motility and fertilization ability of the spermatozoa. Supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryoprotective effect on bull, ram, goat, boar, canine, and human sperm quality, thus improving semen parameters, for example, sperm motility, membrane integrity after thawing (Bucak *et al.,* 2009) [25].

1. Enzymatic antioxidants

Glutathione reductase and glutathione peroxidase

GSH/glutathione peroxidase are the mainly acts as scavenging antioxidants in the epididymis and testes (Mora-Esteves and

Shin, 2013) [59], and helps in preserving sperm viability and motility by confering protection on the lipid constituents of the sperm membrane (Lanzafame *et al.,* 2009) [51]. This property of GSH has been shown in *invitro* studies, wherein the tail-beat frequency was preserved, LPO reduced, and the sperm membrane characteristics were improved (Griveau and Le, 1994) [38].

Superoxide dismutase and catalase: SOD protects the sperm from superoxide anions by catalyzing the conversion of superoxide into oxygen and H_2O_2, and thereby prevents LPO and improves motility (Agarwal *et al.,* 2004)[2]. On the other hand, catalase aids in the decomposition of H_2O_2 into water and oxygen (Mora-Esteves and Shin, 2013) [59], and thus, both SOD and catalase assists in removing ROS that has the potential to damage sperm.

2. Non-enzymatic antioxidants:

Vitamin E

Vitamin E (α-tocopherol) is a chain- breaking antioxidant found in the sperm's cell membrane and acts by neutralizing H_2O_2 and quenching free radicals (Bansal and Bilaspuri, 2011) [18], hence halting chain reactions that produce lipid peroxides and protecting the membrane from the damage induced by ROS (Lampiao, F. 2012) [50]. Furthermore, it also improves the activity of other scavenging oxidants (Mora-Esteves and Shin, 2013) [59] (superoxide, H_2O_2 and hydroxyl radicals) and thus helps to preserve both sperm motility and morphology (Agarwal *et al.,* 2004) [2]. During cryopreservation, vitamin E supplementation has a positive effect on sperm motility, mitochondrial membrane potential, and membrane integrity, depending on the fraction of ejaculate (Cerolini *et al.,* 2000; Pena *et al.,* 2003; Pena *et al.,* 2004) [27, 67, 68]. Addition of vitamin E and selenium (@0.75mg/ml and 2µg/ml respectively) alone in fructose lactose egg yolk glycerol extender has also been reported to have increased the cryosurvival of buffalo sperms (Ali *et al.,* 2017)[6]. Addition of vitamin E (5 µg/Ml) in combined with 1% Nano-Se has also been reported to have improved the post-thawing quality and oxidative variables of rooster semen (Safa *et al.,* 2016) [77]. Also addition of Butylated Hydroxy Toluene (BHT), Pentoxifylline (PTX) and tocopherol (Vit E) in the semen extender has also been reported to have more beneficial effects in the semen quality and preservability of Karan Fries bull's spermatozoa with regards to improved sperm cell function, such as motility, viability, HOST, and acrosome integrity, as compared to the control during liquid storage up to 48 h of preservation at refrigerated temperature. (Bhakat *et al.,* 2011) [21].

Vitamin C

Vitamin C (ascorbate) plays a significant role (up to 65%) in combatting OS in the seminal plasma (Sharma and Agarwal, 1996) [84] by reacting with OH^-, O_2^- and H_2O_2 in the extracellular fluid, thus protecting sperm viability and motility (Lampiao, F., 2012) [50] and prevents sperm agglutination (Agarwal *et al.,* 2004) [2]. However, vitamin C is only a weak ROS scavenger in the cell membrane and, hence, has almost no effect within the cell ((Lanzafame *et al.,* 2009) [51]. It has been reported that the incorporation of both vitamin C and vitamin E into semen extenders has improved the motility and viability of bull and boar spermatozoa (Pena *et al.,* 2003; Jeong *et al.,* 2009) [67, 42].

Carnitine

Carnitine, a water-soluble antioxidant commonly attained from dietary sources. It may participate in sperm motility as a fuel source by assisting free fatty acid utilization and preventing lipid oxidation (Mora-Esteves and Shin, 2013) [59]. Therefore, carnitine protects the sperm DNA and membranes from oxidative damage, and maintains the sperm viability and motility (Sharma and Agarwal, 1996) [83]. L-Carnitine has proven to be effective in chicken sperm protection against apoptosis, mitochondrial activity loss, and DNA defragmentation (Partyka *et al.,* 2017) [63].

In a study it was found that taurine (@ 1 mM to semen extender) provided the best sperm motility, viability, and mitochondrial activity and reduced sperm apoptosis and DNA damage and could be recommended as an additional component of the chicken sperm freezing extender (Partyka *et al.,* 2017) [64]. In the same study, hypotaurine was also found to provide significant increase in sperm motility and protection against LPO.

Albumins

Albumins interacts with peroxyl radicals and prevents the chain reactions that generate more free radicals, hence reducing the ROS production and proserving sperm motility and viability (Sharma and Agarwal, 1996) [84]. They are known to improve sperm motility and plasma membrane integrity, and protect acrosomes from temperature shock during the freeze-thawing of ram semen (Uysal and Bucak, 2007) [91]. Albumins may also promote sperm survival in the reproductive tract of the cow prior to fertilization (Chen *et al.,* 1993)[28], and it has been reported that albumins improve fertility and increase the catalase antioxidant activity following the freeze-thawing of bull semen (Schafer and Holzmann, 2000) [80].

Incorporation of antioxidant additives such as cysteine HCl and ascorbic acid in standard TFYG diluents improves sperm quality parameters, reduces enzyme leakage, and ultimately advances cryopreservability of buffalo semen (Patel *et al.,* 2016) [65]. Vitamin E may directly quench the free radicals such as peroxyl and alkoxyl (ROO •) generated during ferrous ascorbate-induced lipid peroxidation, and is thus suggested as major chain breaking antioxidant (Bansal and Bilaspuri, 2008) [17]. Mn^{2+} enhances sperm motility, viability, capacitation and acrosome reaction by decreasing the oxidative stress (Bansal and Bilaspuri, 2008; Bilaspuri and Bansal, 2008) [17, 22]. Mn^{2+} also promotes the acrosome reaction (Kim and Parthasarathy, 1998)[46] if added extracellular, by enhancing the level of cAMP by stimulating Ca^{2+} or Mg^{2+} ATPase which leads to activation of calcium channel opening, thereby depositing more Ca^{2+} ions.

Zinc is one of the important trace elements in the body deficiency of which causes infertility in most animals due to disorders of testes development, spermatogenesis (Massanyi *et al.,* 2004) [54], steroidogenesis through gonadotropic hormones secretion (Hurley and Doan, 1989) [41], genetic expression of steroid receptors (Freedman, L.P. 1992) [34], testosterone synthesis, serum cholesterol level adjustment (Tajik and Nazifi, 2010) [89] etc. Zinc supplementation leads to improved fertility in zinc deficient animals by increasing concentration and motility of spermatozoa (Cupic *et al.,* 1998) [29] and sperm membrane integrity and reducing sperm DNA damage in human subjects (Omu *et al.,* 2008) [62]. Zinc also contributes to the stability of sperm chromatin and repair of DNA damage (Barratt *et al.,* 2010) [19]. Zinc influences the fluidity of lipids, and thus the stability of biological

membranes. It is involved in the formation of free oxygen radicals and may play a regulatory role in the process of capacitation and the acrosome reaction (Andrews *et al.,* 1994) [10], sperm nuclear chromatin condensation and acrosin activity (Steven *et al.,* 1982) [85].

Though addition of higher zinc concentrations (0.576 and 1.152 mg/L) are detrimental to spermatozoa, Dorostkar et al. (2014) [31] reported that addition of zinc sulphate (0.288 mg/L) improved the sperm quality (progressive motility, viability, membrane integrity and total antioxidant capacity) preservation upon freezing processes and also affected the cell membrane leading to a lower degree of sperm DNA damage after semen freeze-thawing, which in turn, resulted in higher semen fertility.

Amino acids

Amino acids have an important role in preventing oxidative damage to spermatozoa during preservation. Cysteine, a sulphur containing amino acid with thiol group is an important component of nucleic acid presents in head of sperm which maintains the integrity of the DNA. This is also an important component of glutathione which acts as an antioxidant, and being a precursor of intracellular glutathione (GSH), cysteine penetrates the cell membrane easily, enhances the intracellular GSH biosynthesis (both *in vivo* and *in vitro*) and protects the membrane lipids and proteins due to indirect radical scavenging properties. It also has cryoprotective effect on the functional integrity of axosome and mitochondria improving post-thawed sperm motility in many species (Memon *et al.,* 2011) [56]. Addition of cysteine HCl in TFYG and other extender has been reported to result in a higher progressive motility, plasma membrane, acrosomal integrities of different breeds of buffalo bull spermatozoa functional assays (Hazarika *et al.,* 1989; Ansari *et al.,* 2011b; Dhami and Sahni, 1993) [39, 13, 30], though contrary results has also been reported in Surti buffalo bulls (Virani A.C, 1992) [93]. Shannon (1965) [83] demonstrated a factor (probably a heat labile and non-dialyzable protein) in bovine seminal plasma which depressed livability and motility of the spermatozoa. A similar toxic factor was also demonstrated in buffalo seminal plasma (Sengupta *et al.,* 1976) [81], and both these were inactivated by addition of cysteine HCl which was indicated by significant improvement in spermatozoal motility and viability in the presence of the additive. Cysteine HCl has also stimulates aerobic fructolysis and the oxygen uptake by the spermatozoa is maintained for a longer time when semen is stored for a longer time (Sengupta *et al.,* 1969) [82].

The role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen has also been studied, and it has been found that 20 mM "l-glutamine" and 25 mM "l-proline" can be used as semen additive to freeze ram semen as they prevented cryoinjuries to sperm and improved the pre-freeze and post-thaw semen characteristics (Sangeeta *et al.,* 2015) [79]. Also by supplementing the Tris dilution/ refrigeration media with cu 1mM vitamin E or 10mM L-cysteine the quality of refrigerated ram semen has also been reported to have increased (Anghel *et al.,* 2010) [11] Antioxidant additives effect on cytological parameters of refrigerated ram semen. Glutathione not only preserves the anti-oxidant property but also protects the sperm due to its sulfur and cysteine content. Glutathione treated extender has been reported to have a high conception rate (66.00%), and high pregnancies in good 72.00% and poor freezable crossbred bulls 59.00% with artificial inseminations (Perumal, P., 2008) [69]. The addition of glutathione (but not caffeine) has been reported to have a positive effect on fertilizing ability of ram spermatozoa along with the substitution of egg yolk-based extender by a synthetic EquiPro extender for the insemination of ewes without reducing fertility (Kubovičová *et al.,* 2010) [49].

Glycerol or dimethyl sulfoxide can induce osmotic stress and toxic effects to spermatozoa, but the extent of the damage varies according to the species and depends on its concentration in the extender solution (Purdy, P.H., 2006) [71]. Though glycerol appears to provide a protective effect to the acrosomal membrane it has got no overall effect on the plasma membrane (Garner and Thomas, 1999) [35], and increased concentrations of egg yolk in the extender may reduce the glycerol levels (Evans and Maxwell, 1987) [32]. Glycerol is frequently used as a cryoprotectant for freezing ram semen.

Various milk components in semen extenders are known to benefit sperm viability. Native phosphocaseinate is one such component, and has been used as a commercially available product worldwide most popularly as equine semen extenders in the horse industry. It has been found that native phosphocaseinate or casein formulated extenders are superior to an unfractionated nonfat dried milk solids extender in maintaining sperm motility after cooled storage of semen for 24 and 48 hours. In addition, this product has its useful characteristic of lack of debris (which improves clarity for visualizing sperm motility microscopically), when compared with that typically present in nonfat dried skim milk solids extenders (Brinsko and Hartman, 2011) [24].

Freezing media comprised of lipids, specifically phospholipids, have shown much success in limiting damage due to cryopreservation (Buhr *et al.,* 2000; Jiang *et al.,* 2007) [26, 43]. Phospholipid based media have a protective effect on membranes as they have been suggested to replace membrane phospholipids that were damaged or lost during cryopreservation (Buhr *et al.,* 2000; Maldjian *et al.,* 2005) [26, 53] by reducing the effect of cold shock by limiting calcium uptake which has a direct effect on the mechanisms for capacitation (White, 1993) [96]. In addition, membranes strengthened by phospholipids are more able to regulate the flow of ions, proteins, and ATP that are necessary for metabolism and motility (White, 1993) [96].

Egg yolk has been proven to be the most effective based extenders as it contains many lipids similar to those found in sperm membranes (Buhr *et al.,* 2000) [26]. It protects the sperm cell against cold shock and the cell membrane during freezing and thawing due to the presence of phospholipids (lecithin) and low density lipoproteins (Medeiros *et al.,* 2002; Purdy, P. H., 2006) [55, 71]. Egg yolk acts on the cell membrane, having a greater effect in bull than ram spermatozoa. Based on successes with egg yolk based mediums, numerous additives in the form of sugars and buffers are added to control osmolality and pH thus creating extenders such as TEST and BF5 (Graham *et al.,* 1971b; Pursel and Johnson, 1972; Pursel and Park, 1985) [37, 72, 73]. In addition, to aid in the effectiveness of lipid based extenders, if the freezing medium is supplementated with a surfactant (e.g. Ovrus ES Paste.i.e. OEP, comprised of sodium and triethanolamine lauryl sulphate), then it will help in the breaking up of accumulations of lipids, which are then allowed to be absorbed by the sperm and result in improving viability post-thaw (Almlid *et al.,* 1987; Graham *et al.,* 1971a; Pettitt and Buhr, 1998; Pursel *et al.,* 1978) [7, 36, 70, 74].

The seminal enzymes play a crucial role in fertilization, and are affected due to cryopreservation. Thus, the assessment of levels of certain enzymes, *viz*., transaminases, dehydrogenases, in the seminal plasma are very important in judging the preservability and fertilizing capacity of spermatozoa (White, I. G., 1958) [95]. But, seminal plasma, can also be used as another effective additive for cryopreservation medium and has been shown to be beneficial if added in proper proportions prior to freezing (Hernandez *et al.*, 2007) [40], and following thawing (Larsson and Einarsson, 1976; Okazaki *et al.*, 2009) [52, 61]. This characteristics of seminal plasma may be because of its antioxidant like properties, aids membrane function, reverses capacitation (Vadnais *et al.*, 2005) [92], and protecting DNA integrity (Fraser and Strzezek, 2007) [33]. The seminal plasma contains about 65% of the antioxidant capacity in the form of ascorbic acid, and its concentration in seminal plasma exceeds 10 times more than that in blood plasma (364 compared with 40 µmol/L) (Tariq *et al.*, 2015) [90]. Ascorbic acid due to its antioxidant properties prevents the toxic effect to spermatozoa which occur due to hydrogen peroxide release during the cryopreservation and hence the spermatozoa remain viable for a long period but not protected (Sengupta *et al.*, 1969; Mittal *et al.*, 2014) [82, 58]. The bovine spermatozoa motility significantly increases in extender supplemented with ascorbic acid at the concentration of 4.5 mg/ml (Bilodeau *et al.*, 2001) [23] which enhances catalase activity, reduces GSH and GSH peroxidase activity. Similarly, addition of ascorbic acid to egg yolk-lactose glycerol diluent, prior to freezing of bull semen improves the post-thaw sperm motility and survivability (Stolbov and Rimanova, 1984) [86].

It is now known that the ascorbic acid content of buffalo bull semen is significantly lower as compared to cow bull semen (Banerjee and Ganguli, 1973; Reddy and Raja, 1979) [15, 75], which might be the reason for poor *in vitro* preservability of buffalo bull semen.

Antibiotics in semen extenders (Morrell and Wallgren, 2014) [60]

Bacteria have a negative effect on sperm quality, either by directly competing with spermatozoa for nutrients supplied by the semen extender or by the production of toxic metabolic byproducts and endotoxins. Moreover, bacteria may cause inflammation or disease in inseminated females. The female reproductive tract is exposed to bacterial contamination from the male during natural mating and has therefore developed natural defense mechanisms to remove bacteria. However, when AI is used, the semen may be deposited in a non-physiological part of the female reproductive tract, e.g., bull spermatozoa are deposited in the anterior cervix or uterine body during AI instead of in the vagina, and the deposition of semen must occur at the correct time. Moreover, the semen may have been stored in the presence of a nutrient-rich extender that favors the growth of microorganisms. Therefore, antibacterial agents are added to the semen extenders to prevent disease keeping in mind about the antibiotic resistant organisms.

The inclusion of antimicrobials in semen extenders is required by both national and international regulations to control the microbial content. Although frozen semen retains its microbiological profile during storage, it is susceptible to deterioration on thawing; fresh and cooled semen is likely to deteriorate during storage and transport. The antibiotics to be added and their concentrations for semen for international trade are specified by government directives, and since the animal production industry uses large quantities of semen for artificial insemination, large amounts of antibiotics are currently used in semen extenders especially in foreign countries.

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