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Antioxidant and spermatozoa: a complex story- A review

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ABSTRACT

The artificial insemination is a common practice in domestic animals, but the use of frozen semen compromises the fertility of goat spermatozoa based on pregnancy rates from AI. This could be due to a poor resistance of goat spermatozoa to cryopreservation stresses compared to sperm from other domesticated mammals. Lipid peroxidation caused by reactive oxygen species limits the success in this species. Reports revealed that inclusion of antioxidants in cryopreservation media improved quality of semen against LPO. Moreover, inclusion of enzymatic and non-enzymatic antioxidants (vitamin C, glutathione reduced etc) in diluents at appropriate concentration can help in holding the semen at refrigeration temperature for many days.

Key words: Antioxidants, Cryopreservation, Reactive oxygen species, Semen

Cryopreservation leads to significant reduction in the level of spermatozoa antioxidants and this generates reactive oxygen species (ROS) such as superoxide radical (O_2), hydroperoxyl radical (HO_2) and hydroxyl radical which can cause cell damage. Excess production of reactive oxygen species during cryopreservation is associated with reduced post-thaw motility, viability, membrane integrity, antioxidant status, fertility and sperm functions. The addition of antioxidants into semen extenders improved semen quality in boar (Satorre *et al*. 2007), bull (Kumar 2007), stallion (Almeida *et al*. 2005), dog (Michael *et al*. 2007). However, there are not much reports on this aspect in goat. Therefore the present work highlights the measures of semen quality, importance and role of antioxidants in minimizing the aforesaid changes in goats, which have defense mechanism against lipid peroxidation of semen and are important in maintaining sperm motility, viability and DNA integrity in goat (Saraswat *et al*. 2012, 2014).

Measures of semen quality: Semen production and evaluation of semen quality attributes of breeding bucks constitute an important basis of predicting the *in vitro* and *in vivo* fertilizing ability of spermatozoa (Kumar 2007). With the widespread interest in the application of AI in domestic animals, there is a growing interest and necessity for more knowledge concerning the reproductive characteristics of farm animals. Morphological evaluation of spermatozoa must be conducted to ensure semen of high quality. It is well known that ejaculate of goat bucks is quite small in volume (0.7 to 2 mL). Extensive observations on the volume of semen in different goat breeds revealed varying results. Thakur *et al*. (2005) reported non-significant variation reported among Chegu bucks with the mean volume (0.47±0.03 ml). Peterson *et al*. (2007) recorded mean semen as 1.3±0.5 ml of Sirohi buck semen cryopreserved under different freezing modes and Saraswat *et al*. (2012) as 1.05±0.06.

Colour and consistency: The colour and consistency of a semen sample depends on the number of spermatozoa per milliliter of the semen. Colour varies from white to milky or creamy white. Yellow colour is the indicator of more riboflavin. Brownish colour is indicative of most dead spermatozoa. Consistency of semen may be thick or thin. Thick consistency is associated with high sperm concentration (Saraswat *et al*. 2012)

Mass activity: Mass activity of wave motion characteristics in neat semen depends upon concentration of progressive motile spermatozoa and speed of progression of spermatozoa. Exposure of ejaculated semen to low or higher temperature, chemicals or foreign agents reduces the motility. Mass activity is influenced by breed, age and individual (Srinivas *et al*. 2002). Thakur *et al*. (2005) evaluated the semen production potential of Chegu bucks and reported that the mass activity of neat semen varied significantly among bucks that ranged from 3.67±0.33 to 4.78±0.15 on 0–5 scale. While Mara *et al*. (2007) studied the effect of different diluents on goat semen fertility and reported that mass activity at initial storage was 3.6±0.2 on 0–5 scale, however in Sirohi buck semen; Saraswat *et al*. (2012) reported 3.98±0.03 on 0–5 scale.

Progressive motility: Motility, a physical characteristic of spermatozoa associated with flagella, is due to contractile

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substance of axial filament of the spermatozoa midpiece, where the wave of action starts and through which it precedes the length to the entire tail. The spermatozoa do not move at random in all directions independently of each other but in waves. When a fairly thick layer of semen is examined under the low power objective microscope, the interplay of these waves can be observed as a swirl over the field of focus. As the motility is initiated, the spermatozoa tend to move in the direction of their head point, thus sperm orientation. In addition, motility is an essential feature of healthy spermatozoa and studies showed that sperm motility is essential for normal fertilization and it is currently the most common parameter of sperm quality acting as an indirect measure of metabolic activity and sperm viability (Berlinguer *et al*. 2009). Sperm motility after thawing and washing provided the most significant information for predicting donor sperm fertility potential, compared with fresh and thawed specimens used for the insemination. The plasma membrane of sperm is the primary site of damage induced by cryopreservation resulting in reduced motility and fertility of sperm cells after cryopreservation. The low fertility of cryopreserved semen samples may well be due to decrease in the number of normal sperm in samples. In other words, when plasma membrane is exposed to low temperatures, cold shock in cooling step and cryo-damage in freezing step cause the irreversible loss of motility during cryopreservation (Bucak *et al*. 2010, Saraswat and Jindal 2011). Saraswat *et al*. (2012) reported maximum (78.19±0.94%) and minimum (14.44±4.29%) progressive motility in Sirohi buck semen.

Hypo-osmotic swelling test (HOS test): Proper structural integrity of spermatozoa membrane is essentially required to maintain fertilization, therefore an important property of sperm cell membrane is its ability to permit selective transport of molecules. When exposed to hypo-osmotic conditions, water will enter the spermatozoon in an attempt to reach osmotic equilibrium. This inflow of water will increase sperm volume, and the plasma membrane will bulge (balloon). It can be assumed that ability of the sperm tail to swell in the presence of a hypo-osmotic solution indicates that the membrane is intact. Freezing and long term storage makes sperm plasma membrane susceptible to peroxidative damage with consequent loss of membrane integrity, resulting from reactive oxygen species. Integrity of the sperm plasma membrane is essential for cell survival and fertilizing ability. The hypo-osmotic swelling test (HOS) has been applied to goat (Ranjan *et al*. 2009). HOS evaluates the functional status of the sperm plasma membrane. The principle of the test is based on water transport across the sperm membrane under hypo-osmotic conditions. During the HOS test, spermatozoa with intact and functionally active plasma membranes undergo swelling (due to the influx of water) and subsequently increase in volume to establish equilibrium between the extra- and intracellular compartments. Use of this inexpensive and simple assay was recommended as an additional fertility indicator parameter (Saraswat *et al*. 2012).

Acrosomal status: The acrosome is a membrane enclosed structure covering anterior part of sperm nucleus. This structure contains powerful hydrolyzing enzymes and is a basic feature of the sperm head of all eutherian mammals. As a prerequisite for normal fertilization, the content of the acrosome is released into its surroundings during the acrosome reaction (AR) for the successful penetration of the oocyte (Rijsselaere *et al.* 2005).

Evaluation of acrosomal status is an essential criterion in the evaluation of quality of a semen sample prior to its use for artificial insemination (AI). Presence of an acrosomal cap is important in fertilization process and is highly related with fertility of frozen semen (Saraswat *et al*. 2012). Since cooling and freezing involves oxygen damage during preservation so a decrease in the number of morphologically normal sperm in ejaculates leads to reduced fertility of the cryopreserved semen samples. The incorporation of the antioxidants (ascorbic acid, reduced glutathione, oxidized glutathione and alpha tocopherol, etc.) in semen diluter resulted in higher percentages of normal acrosome (Saraswat *et al*. 2011, 2012). Thus, this functional test provides valuable knowledge about sperm function, physical parameters of goat semen to the quality of seminal dose after freezing, to test whether measurements made after ejaculation would be useful for predicting sperm quality of frozen–thawed samples.

Live spermatozoa: The percentage of live spermatozoa presents an important criterion in evaluation of semen and can be estimated by visual appraisal and by the so-called vital stains. Vital different staining techniques are used for counting live and dead spermatozoa in semen smears, however, eosin-nigrosin stain is widely used for this purpose. In this staining technique, eosin stains the dead spermatozoa as pink or red whereas live spermatozoa remain colorless, as they are impermeable to the eosin stain. Nigrosin provides a blue–black background. Saraswat *et al.* (2012) studied the effect of antioxidants addition on Sirohi buck semen and found that ascorbic acid at 9mM and reduced glutathione at 7mM concentration had highest live sperm in frozen and diluted semen.

Cryopreservation: Semen cryopreservation plays a key role in propagation of germplasm, and is being applied for many animals including both wild and domestic animals. The improvement of semen cryopreservation techniques requires in-depth knowledge of gamete physiology and biochemical processes occurring during semen collection, processing, and freeze-thawing. Freezing/thawing of sperm sample is routinely conducted to perform artificial insemination. These procedures (cryopreservation) produce reactive oxygen species (ROS) or free radicals in sperm samples. During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which increases their susceptibility to lipid peroxidation (LPO). Moreover excessive production of these reactive oxygen species (ROS), impairs the motility and fertilization capacity (Saraswat *et al*. 2013).

Physiology and effects of cryopreservation: Both freezing and thawing cause tremendous alterations in cell

water volume. Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation and lack the significant cytoplasmic component containing antioxidants that counteract the damaging effect of reactive oxygen species and lipid peroxidation (Bucak *et al.* 2008). Owing to this, spermatozoa are susceptible to lipid peroxidation (LPO) during cryopreservation and thawing which confers considerable mechanical stress on the cell membrane. Long-term (freezing) and short-term (liquid) storage of sperm may lead to plasma membrane deterioration due to membrane phase transition occurring in the regions of highly specialized sperm plasma membrane (Saraswat *et al.* 2012, Bucak *et al.* 2009). Antioxidant capacity of semen may however be insufficient in preventing lipid peroxidation during the freezing thawing process. The mammalian sperms lack a significant cytoplasmic component, which contains sufficient antioxidants to counteract the damaging effects of ROS and LPO. For this reason, sperm are highly susceptible to lipid peroxidation during cryopreservation and thawing process resulting in sub lethal damage. Membrane leakiness, DNA fragmentations, mitochondrial damage are the possible outcomes of free radical damage. Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease the fertility potential of the cells. Procedure of cryopreservation increases premature capacitation of spermatozoa as freezing and thawing processes lead to the generation of reactive oxygen species (ROS). Excessive production of ROS during cryopreservation has been associated with the reduced post thaw motility, viability, membrane integrity, antioxidant status, fertility and sperm functions (Uysal and Bucak 2007). Sperm plasma membrane is one of the key structures affected by cryopreservation (Agarwal *et al.* 2004).

Causes of sperm damage and oxidative damage: Sperm cryopreservation involves several steps such as centrifugation, sperm dilution, cooling, freezing and thawing. Each of these steps can cause sperm damage which impairs normal sperm function and fertilizing potential. The main causes of sperm damage during cryopreservation are classified as (i) cold-shock, (ii) osmotic stress and (iii) oxidative stress (Saraswat 2011). Cooling before the freezing process causes stress to the spermatozoa due to the temperature changes that result in sperm damage known as cold shock. Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly known as reactive oxygen species (ROS). Apart from molding of membranes, it plays an important role in dynamic status that affects the fusion of the plasma membrane of the male and female gametes. Mammalian spermatozoa are known to be sensitive to oxidative damage. During sperm cooling, freezing and thawing, the excessive metabolites of oxygen such as ROS can cause cell damage or apoptosis. Several studies have shown that oxidative stress induces sperm membrane and DNA damage in human (Agarwal *et al.* 2003), stallion (Baumber *et al.* 2003), ram (Peris *et al.* 2007) or bull spermatozoa (Bilodeau *et al.* 2002).

What are reactive oxygen species (ROS): ROS are partially reduced forms of atmospheric oxygen (O_2) or are highly reactive oxidizing agents belonging to the class of free radicals. Free radical is any compound containing one or more unpaired electrons. The unpaired electron finds other electrons to become paired. Thus, the free radicals always attack other molecules to complete their unpaired electron. Reactive oxygen species (ROS) result from the excitation of O_2 to form singlet (O_2^1) or from the transfer of one, two or three electrons to form a superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($OH^\cdot$) respectively (Saraswat *et al.* 2013). Excessive ROS can be generated by lack of antioxidants in seminal plasma and spermatozoa, normal and immotile abnormal spermatozoa (Silva 2006).

Types of reactive oxygen species: ROS represent a broad category of molecules that indicate the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives. Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion, etc.) are free nitrogen radicals and considered a subclass of ROS (Saraswat *et al.* 2013).

Physiology and action of ROS: Mammalian sperm plasma membrane, rich in polyunsaturated fatty acids (PUFA) can be easily damaged by the reaction between ROS such as hydroxyl radicals ($^\bullet OH$) and the polyunsaturated fatty acids. This mechanism is widely known as the lipid peroxidation reaction. Lipid peroxidation of sperm membrane is considered to be the key mechanism of ROS induced sperm damage leading to chromatin destabilization associated with marked alterations in the DNA-protein complex. It also affects the functions of various enzymes, including cytochrome oxidase, lactase dehydrogenase and glucose-phosphate dehydrogenase. Furthermore, ROS distrupts mitochondria functions, inhibit the synthesis of DNA, RNA and proteins, increase DNA fragmentation, modify the cytoskeleton, affect the axoneme through the oxidative stress and the production of cytotoxic aldehydes, composed of three major steps: initiation, propagation and termination. The initiation step is the process of producing lipid radicals by ROS. In the propagation step, the lipid radicals from the initiation step attack other unsaturated fatty acid molecules on the cell membrane or steal electrons from oxygen (O_2) to become hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) can continue to attack other polyunsaturated fatty acids (PUFA) on the cell membrane. This step can continue so the lipid peroxidation reaction is also known as the chain reaction. In the termination step, which is the end stage of the lipid peroxidation reaction, there is a combination of free radicals to form paired stable electrons. This step can be stopped earlier by antioxidants that can trap free radicals. The lipid peroxidation reaction results in changes in sperm membrane fluidity, loss of membrane integrity as well as irreversible loss of sperm motility. Lipid peroxidation is also related to

DNA strand breaks (Baumber *et al*. 2003). Protection of these processes against the harmful effects of reactive oxygen species (ROS) is provided mainly by antioxidant enzymes (Koziorowska-Giluna *et al*. 2015).

Strategies to reduce oxidative stress: Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa, to prevent oxidative damage. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility. Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl (ROO·) generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant (Saraswat *et al*. 2012). Thiol groups also play an important role in detoxification and antioxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defense mechanisms of sperm cells to fight against oxidative stress. A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation. Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants had shown to provide a cryoprotective effect on bull, ram, goat, boar, canine and human sperm quality, thus improving semen parameters (sperm motility, membrane integrity) after thawing (Bucak *et al*. 2010, Saraswat *et al*. 2012). Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique for the goat breeding industry.

Physiology and effect of antioxidants on oxidative stress and sperm functions: During the sperm preservation process, spermatozoa are generally stored under aerobic conditions. Spermatozoa require oxygen to support and maintain their normal cell functions and metabolism. The excessive oxygen metabolite products such as reactive oxygen species (ROS) are, however, considered as a cause of sperm damage such as loss of membrane fluidity, decrease in motility and increase in DNA breakage. Therefore, one absolute requirement for the use of cryopreserved sperm is that the cryopreservation process must not induce alterations that would impair progeny development. Altered spermatozoa are known to produce reactive oxygen species in particular after cryopreservation. These peroxidation products are highly deleterious for DNA and can induce strand breaks and base modifications as well as progeny viability and development. These peroxidation products were eliminated by the addition of antioxidative enzyme or non-enzymatic antioxidant to semen preservation media to improve chilled and post-thaw sperm longevity and quality in several animal species (Funahashi *et al*. 2005).

Types and importance: The antioxidants are classified into two categories due to their function: enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are also known as natural antioxidants; they neutralize excess reactive oxygen species (ROS) and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) which also causes reduction of hydrogen peroxides to water and alcohol. The function of enzymatic antioxidants is to suppress the formation of free radicals by decomposition of the free radicals. SOD protects spermatozoa against spontaneous O_2 toxicity and LPO. SOD and catalase also remove O^-_2 generated by NADPH oxidase in neutrophils and play a major role in decreasing LPO and protecting spermatozoa against oxidative damage. Catalase presence in sperm has been demonstrated for ram and cattle and it has a potential role in ageing process and control of oxidative stress in cells, mainly resulting from H_2O_2. Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione (Agarwal *et al*. 2005). Nogushi and Niki (1999) called non-enzymatic antioxidants as radical scavenging antioxidants and classified these non enzymatic antioxidants into two sub-types: hydrophilic and lipophilic antioxidants. The hydrophilic (water soluble) antioxidants, such as glutathione existing under its reduced (GSH) and oxidised form (GSSG), uric acid, ascorbic acid (vitamin C), thiols, proteoglycans and hyaluronic acid, protect against lipid peroxidation as well as against protein, carbohydrate and nuclear material oxidation. On the other hand, lipophilic antioxidants mainly composed by α-tocopherol (vitamin E), β-carotene (vitamin A), flavonoids, ubiquinol (Coenzyme Q10), bilirubin and melatonin, and are essentially protecting lipids from chain reactions of peroxidation. The function of non-enzymatic antioxidants is to inhibit chain initiation and break chain propagation stages in the lipid peroxidation reaction.

Antioxidants proved to be beneficial in protecting damaging effects of ROS on sperm movement and against oxidative damage such as glutathione are a molecule found at mM level in a number of cells and is able to react with many ROS directly. GSH is also a cofactor for glutathione peroxidase (GSHPx) that catalyzes the reduction of toxic H_2O_2 and other hydroperoxides, protecting the mammalian cells from oxidative stress. Glutamine (5mM) has cryoprotective effect by improving post thaw motility, membrane integrity and catalase enzyme activity in ram semen (Bucak *et al*. 2009). Supplementation of inositol in the extender can improve the motility of frozen thawed bull sperm (Bucak *et al*. 2010). Inositol has cryoprotective and antioxidative properties resulting in higher antioxidant GSH activity, acrosome integrity, and intact morphological rates (Bucak *et al*. 2010). Cysteine is a low-molecular weight amino acid containing thiol; it is a precursor of intracellular glutathione (Uysal and Bucak 2007). It penetrates the cell membrane easily, enhancing the intracellular GSH biosynthesis both *in vivo* and *in vitro* and protecting the membrane lipids and proteins due to indirect radical scavenging properties (Traber and Atkinson 2007). Trehalose or taurine, a sulfonic amino acid, acts as non-

enzymatic scavenger that plays an important role in the protection of spermatozoa against ROS, in case of exposure to aerobic conditions and the freezing-thawing process. A non permanent disaccharide has a protective action related both to osmotic effect and specific interactions with membrane phospholipids, rendering hypertonic media, causing cellular osmotic dehydration before freezing and then decreasing the amount of cell injury by its crystallization. Trehalose performs better cryoprotection post thaw fertilizing ability in ram, bull, and mouse sperm due to diminished death and damage of sperm (Bucak *et al*. 2007). Hyaluronan, an essential component of the extracellular matrix and non sulfated glycosaminoglycan, is involved in important physiological functions such as motility, capacitation of spermatozoa and preserve post thaw spermatozoa viability and *in vitro* membrane stability. Hyaluronan improves sperm motility, viability and membrane integrity after freezing and thawing procedure and decrease polyspermy with declining motility in humans and boars (Bucak *et al*. 2007). Bovine serum albumin (BSA) is known to eliminate free radicals generated by oxidative stress and protect membrane integrity of sperm cells from heat shock during freezing thawing. Carotenoids such as beta-carotene and lycopene are also important components of antioxidant defense. Addition of antioxidants such as vitamin E, butylated hydroxytoluene (BHT), and tempo to extended turkey semen improves sperm survival and membrane integrity and reduces the loss of motility after 48 h of storage (Uysal and Bucak 2007).

Antioxidant defense mechanisms in semen: Antioxidant defense mechanism in semen includes three level of protection. *Prevention*: Prevention of ROS formation is the first line of defense against oxidative stress. An example is the binding of metal ions, iron and copper ions in particular, which prevents them from initiating a chain reaction. *Interception:* To break chain reaction by formation of non-radical end products because “radical begets radical”. For example α-tocopherol (vitamin E) which is a chain breaking antioxidant, produce tocopheryl radicals during its oxidation, which can then be reduced by ubiquinone or by ascorbic acid. The oxidation of vitamin C gives rise to ascorbyl radicals which can be reduced by glutathione, producing thiyl radicals and oxidized glutathione. This last step can be reversed by glutathione reductase. The defense system of enzymatic antioxidants indicates that catalase (CAT) is a hydrogen peroxide (H_2O_2) detoxifier and also gluthathione peroxidase (GPx) decomposes hydrogen peroxide (H_2O_2) to water and glutathione disulphide which is an important cellular antioxidant. The defense system of non-enzymatic antioxidants indicates that α-tocopherol is the most important lipid-soluble antioxidant, and that it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidised α-tocopheroxyl radicals produced may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol. While ascorbic acid on oxidation decompose to form DHAA, ascorbyl radical and H_2O_2, DHAA and ascorbyl radicals on reduction and in presence of glutathione reductase form thiyl radicals and hydrogen peroxide form oxidized glutathione. *Repair*-Damage caused by oxidants may be repaired by fortification of extender with antioxidant. From the aforesaid study, it is clear that non-enzymatic antioxidants are beneficial in protecting damaging effects of ROS on sperm movement and against oxidative damage caused due to cryopreservation. So, the use of above antioxidants (reduced glutathione, ascorbic acid and α-tocopherol) can reduce the loss of post thaw progressive motility, damage to sperm survival and membrane integrity (Saraswat *et al*. 2012) after long term storage in liquid nitrogen (–196°C).

Glutathione reduced (GSH) as an antioxidant in semen extender: Glutathione (L-alpha glutamyl L-cysteine glycine, GSH) is a tripeptide ubiquitously distributed and plays an important role in the intracellular defense mechanism against oxidative stress. Sperm cells lack reduced glutathione, where one of its functions is to protect cells against the destructive effect of ROS. Mammalian sperm vary, but tends to be low in GSH, glutathione peroxidase (GSP) and glutathione reductase (GPD), the key components in this protective system. One of its characteristic features is that it is a glutathione-dependent enzyme. Glutathione is a crucial antioxidant that can protect mitochondria due to the presence of free radical, can control homeostatic both inside and outside the cells. As it is a sulfhydril antioxidant (SH), an antioxidant and enzyme cofactor which can neutralize free radical, the addition of glutathione as a primary antioxidant is expected to reduce the damage of plasma membranes. The basic function of GSH in mammalian semen is related to its interactions with other systems as a preventive mechanism against reactive oxygen species (ROS). This scavenging function of GSH helps to counteract the effects of oxidative stress in sperm cells, which could result in lipid peroxidation of plasmalemma, irreversible loss of motility, leakage of intracellular enzymes and damage of the chromatin. Addition of glutathione to the semen extender prevents loss of sperm motility by inhibition of lipid peroxidation caused by ROS in frozen thawed goat semen (Saraswat *et al*. 2012). Uysal and Bucak (2007) observed the effect of various antioxidants on the quality of frozen thawed ram semen. Ejaculates were diluted with CTR extender and supplemented with GSH (5mM) and GSSG (5mM), the antioxidant reduced glutathione (GSH) at 5mM had a significant effect in maintaining sperm motility (71.0±3.1) and membrane integrity (79.3±3.7) as compared to oxidized glutathione (GSSG) (5mM). Similarly, Saraswat *et al*. (2012) in frozen thawed goat semen observed that supplementation of reduced glutathione at 7mM concentration in extender increased progressive motility, live sperm and intact acrosome.

Ascorbic acid as an antioxidant in semen extender:

Ascorbic acid is a water soluble vitamin (commonly named as vitamin C) which has been shown to have antioxidant properties. Since cooling and freezing of semen involves oxygen damage during preservation, several studies have been carried out to study the effect of vitamin C addition to diluent with emerging results. Addition of this antioxidant (ascorbic acid) in semen diluent improve the motility during liquid semen storage at 5°C (Ball *et al*. 2001) and also prevent the damage induced by free radicals during freezing and therefore improve fertility of liquid ram spermatozoa. It neutralizes hydroxyl, superoxide and hydrogen peroxide (H_2O_2) radicals and prevents sperm agglutination. It helps in protecting sperm from reactive oxygen species and in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA. Saraswat *et al*. (2012) in goat semen observed that supplementation of ascorbic acid (9mM) is capable to protect sperm from reactive oxygen species, lipid peroxidation and DNA damage.

α-Tocopherol as an antioxidant in semen extender: Vitamin E is a family of α^-, β^-, γ^-, and δ^- (respectively: alpha, beta, gamma, and delta) tocopherols and corresponding four tocotrienols. Vitamin E is a fat-soluble antioxidant that stops the production of reactive oxygen species formed when fat undergoes oxidation. The principal chain breaking antioxidant vitamin E is present within the cell membrane; it neutralizes H_2O_2 and protects the plasma membrane from lipid peroxidation. Vitamin E is classified as the most potent non-enzymatic antioxidant, can inhibit the propagation stage of lipid peroxidation in the membranes by scavenging peroxyl ($RO^{\cdot}$)/ ($LOO^{\cdot}$), alkoxyl ($RO^{\cdot}$)/($LO^{\cdot}$) and other lipid derived radicals. The α- tocopherol radical formed is believed to be reduced by ascorbic acid (Vitamin C) to regenerate Vitamin E. Alpha tocopherol is a well recognized inhibitor of lipid peroxidation in biological membrane and demonstrate a protective effect on the plasma membrane of the bovine spermatozoa during cryopreservation. It can also break the covalent links that ROS have formed between fatty acid side chains in membrane lipids, thus plays an important role in reducing membrane damage caused by excessive ROS production during cryopreservation (Saraswat *et al*. 2012). Vitamin E reacting with lipid peroxyl radicals is formed to be vitamin E radicals but this free radical form is however, stable because the free electron is delocalized to be an aromatic ring structure. Thus, the radical reaction chain of lipid peroxidation is stopped. Saraswat *et al*. (2012) in Sirohi buck semen observed that supplementation of α-tocopherol (4.5mM) is capable to protect sperm DNA from damage.

GAP in Indian context

Cryopreservation of semen was carried out using Tris based extender containing 10% eggyolk and 6% glycerol. The conception rate (CR) of AI using frozen semen (without antioxidants) in goat was 23.3% in Sirohi, 25.9% in Barbari and 30% in Jakhrana breeds (Annual report, CIRG, 2011–12), however 53.12% pregnancy rate was obtained in Jamunapari goats with CR of 28.57% (Annual report, CIRG, 2012–13). Reports are negligible that limits the success in this species by the inclusion of antioxidants in the cryopreservation media. The use of enzymatic and non-enzymatic antioxidants in semen diluter might help to attain CR upto 80%.

Future perspectives

To achieve the better fertility rate, the research must continue developing new techniques of cooling that prevent lipid peroxidation, oxidative stress caused by reactive oxygen species during semen freezing. Researchers must be very aware of technologies of freezing process that causes stress to the spermatozoa due to the temperature changes and results in sperm damage.

This review highlights the impact of antioxidants on goat spermatozoa to cryopreservation that limits the success in this species. Further, the review addresses many aspects of sperm protection, sperm motility, viability and membrane stabilization of sperm cells during relative low temperature storage. Various enzymatic and non-enzymatic antioxidants are available that play an important role in protecting sperm from free radical species during cryopreservation. Thus the need of hour is to improve the current techniques for processing, cooling, packaging and freezing semen with the use of antioxidants. This may reduce the oxidative stress and enhance the viability and fertility of goat spermatozoa.

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