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Review

OXIDATIVE STRESS IN RAM SEMEN AND ANTIOXIDANT TREATMENT

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ABSTRACT

The oxidative stress constitutes accumulation of free radicals (FR) in the mammalian cells and tissue. Elevated accumulation of reactive oxygen species (subset of free radicals that contain oxygen) lead to a number of pathological abnormalities in cellular functioning respective to cells and organ damage.

Excessive reactive oxygen species (ROS) formation can induce significant changes in sperm morphology, vitality, and oocyte fertizability. Spermatozoa membrane is composed of polyunsaturated fatty acids, which undergo oxidation under the action of free radicals. These processes result in reduced motility and impaired ability of sperm to fertilize the ovum.

The antioxidants are the natural inhibitors of ROS activity. In the semen, they are found in large quantities in the seminal plasma. Their biological role is to attenuate the damaging effects of ROS. Low amounts of ROS are involved in the normal physiological and metabolic processes of the animal organism. Various reasons related to rearing, feeding and exploitation of the male animals can cause their overproduction. The imbalance between natural antioxidants and ROS production and accumulation result into body falling into the so-called oxidative stress (OS). This effect negative on various cell organelles. Damage to the sperm plasma membrane is caused by an imbalance between reactive oxygen species and antioxidants, which occurs especially during the semen processing (dilution, freeze-thaw).

The aim of this literature review is to summarize the mechanism of influence of the oxidative stress and the possibilities to reduce its harmful effect over the ram semen parameters after chilling or freezing process.

Key words: oxidative stress, ram semen storage, antioxidants

INRODUCTION

The ewes are seasonally polycyclic animals (1) with the breeding season beginning in summer or early autumn in most sheep breeds (2). Its duration may vary according to latitude, breed and feeding of animals but generally ending in the winter (3). The non-breeding season in ewes is connected with an interruption of the ovarian folliculogenesis, but the rams can be used year-round (4, 5). The season significantly affect sperm volume, concentration, motility, viability, morphology and DNA integrity (6-9). The degree of reproductive regression varies among breeds, but the testicular function and semen quality are reduced during anestrus (5, 10, 11).

Sexual maturity in ram lambs occurs on average at 6-9 months of age (12), but the breeding age is when they reach 65% of the body weight of adult animals of the respective breed (13). The full spermatogenetic cycle needs 47 days, which requires preliminary preparation of the male animals for mating or artificial insemination (12). It includes appropriate feeding, ensure of normal zoohygienic conditions, prevention of heat stress and traumas, necessary for production of high quality semen from proven males (13, 14). The inclusion of rams in the breeding plan requires preliminary clinical examination of each male animal and complete evaluation of the semen before its use as fresh,

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chilled or frozen (15). This evaluation is also mandatory in animals that have previously shown good semen performance but after that were sick, and in the case of low conception rate post mating or artificial insemination.

MECHANISMS OF OXIDATIVE STRESS IN RAM SEMEN

The semen of small ruminants is particularly sensitive to temperature changes. Insemination by fresh semen is the most widely applied, while results are inconsistent when cooled and frozen semen has been used (16). One of the main causes of spermatozoa damage is the accumulation of free radicals due to oxidative stress (OS) in the body. Garrido et al. (17) highlighted two main sources of free radicals in sperm - leukocytes and immature spermatozoa. Oxidative stress causes sterility by several mechanisms:

1. ROS damages the sperm membrane (containing a large number of polyunsaturated fatty acids, which are vulnerable to ROS attacks by the so-called lipid peroxidation), leading to decreased motility and difficult fusion between sperm and oocyte (18-20).

2. ROS mitochondrial damage, which reduces the energy available in the cell and thus impedes the movement of sperm (21-23). In impaired motility, a smaller number of sperm reaches the egg, which in turn greatly reduces the likelihood of fertilization (24, 25).

3. ROS damages sperm DNA.

In the antioxidant protection of the semen, are included three main enzymes (Antioxidant enzymatic defense system):

a) Superoxide dismutase (SOD) - metalloenzymes that catalyze the dismutation of two superoxide radicals to O2 and H2O2;

b) catalase (CAT) with major detoxifying effect;

c) glutathione peroxidase (GSH-Px) converting hydrogen peroxide and lipid peroxides into harmless molecules.

In addition to enzymatic antioxidant systems, other non-enzymatic components (Vit. C, E, D, urates, albumins, taurine and glutathione) are present in the semen and act as ROS inhibitors (26).

IMPROVING THE SEMEN QALITY DURUING STORAGE BY SUPLEMENTATION THE ANTIOXIDANTS

The development of assisted reproductive technologies provides a possibility for different techniques for storage of semen from genetically valuable animals to be utilized (27). The main methods of semen preservation in liquid state are storage at low (0-5 or 10-15°C) and at ambient temperatures by reversible inactivation of spermatozoa (28). Long-term storage of ram semen is accomplished by deep-freezing in a liquid nitrogen. This procedure requires special equipment, but spermatozoa survival after thawing is relatively low, because of high sensitivity of the sperm membranes to freezing/thawing processes (29). After freezing, spermatozoa are more susceptible to ROS-induced damage, and frozen spermatozoa have reduced antioxidant activity (30-32). During freezing, the farm animals' semen can be influenced by oxidative stress, which affects sperm plasma membrane function and sperm motility (29, 33-35).

A great number of antioxidants have been found in seminal plasma. In prolonged storage of chilled or frozen semen, the plasma membrane is affected and the protective ability against ROS generation is significantly reduced (36). The spermatozoa contain a small amount of antioxidants in their cytoplasm (37). That was a reason of various synthetic antioxidants to be used in the different protocols for chilling or freezing of ram semen (38-42). The available studies don't impose a "gold standard" in the composition of semen diluent for handling and storage of small ruminant semen. It confirms the need for further investigations on the oxidative stress and antioxidant capacity of ram semen in relation with determination of the biochemical changes during semen storage, and *in vivo* and *in vitro* evaluation of sperm fertility. Regardless of dilution rate, temperature or storage conditions, semen quality decreases with increasing storage time. The main changes occurring during the extended storage include a decrease in sperm motility and morphological integrity (43). These changes may be caused by the accumulation of toxic products of metabolism mainly reactive oxygen species formed by lipid peroxidation of sperm membranes (28, 44-47).

Semen freezing provides significant advances in harnessing genetic potential and increasing reproductive efficiency in animal husbandry (48, 49). In order to improve the quality of ram semen in long-term (chilled/frozen) storage, various antioxidants or antioxidant compounds (enzymes, amino acids, vitamins, plant extracts), seminal plasma, sugars, fatty acids

and nanoparticles have been added to some semen extenders (50). Many authors report for implementation of antioxidant substances for the sperm cells protection or damage reduction, improve of the survival rate after thawing and high fertility maintenance (38-42). A number of studies have been conducted to investigate the effects of antioxidants on semen quality, but the results are rather variable (51).

In spite of the general agreement for the negative effects of oxidative stress on the spermatozoa (52, 53) and the beneficial effect of the antioxidants on semen quality (54, 55), some authors have reported marginal improvement or even deleterious influence of the antioxidant supplementation in sperm extenders (50, 54-56). Mata-Campuzano et al. (51) found that most of the administered antioxidants (dehydroascorbic acid, TEMPOL, N-acetyl-cysteine, and rutin) had a suppressive effect on sperm motility, although most of them were effective in removing free radicals and protecting DNA and membranes from oxidation. Vitamin E is one of the most important lipid-soluble primary protective antioxidants. Its main role as an antioxidant is thought to be in chain breaking by scavenging lipid peroxyl radicals that propagate lipid peroxidation (57). The group of lipid-soluble compounds (tocopherols and tocotrienols) acts as antioxidants and protects the body from oxidative stress (58). It is known that Cysteine protects spermatozoa from toxic oxide metabolites that induce LPO of sperm plasma membranes under *in vitro* conditions (59). Todorov and Todorova (58) conducted a study to establish the influence of the antioxidant supplements Cysteine and Vit. E on the standard parameters of ram semen cooled at 5ºC. Their result were indicative for an improvement of the sperm cells quality after adding 1mM vit. E or 10mM L-cysteine to Tris buffer. Lycopene is the most plentiful carotenoid in tomatoes, red fruits and is considered to be the best effective antioxidant of all carotenoids (60). Lycopene supports the protection of cells and tissues against the detrimental effects of lipid peroxidation. The antioxidant activity of lycopene is almost catalytic (61, 62). The mechanism of lycopene action involves quenching of singlet molecular oxygen and trapping of peroxy radicals. Cysteamine illustrates its antioxidant and antiapoptotic effects by inducing intralysosomal cysteine accumulation (63, 64). Peker Akalin et al. (65) investigated the effect of different doses of lycopene and cysteamine on chilled ram semen and reported that the use of 0.5 mM lycopene increased mitochondrial activity at 72 h of semen storage. Supplementing 2 mM lycopene at 0 h, 1 and 2 mM cysteamine at 48 h or 1 mM cysteamine at 72 h increased Total Glutathione (tGSH). Also, 1mM cysteamine added at 48 h improved sperm motility. Bezerra et al. (66) studied the effect of different concentrations of adenosine on the quality of chilled ram semen. Adenosine in concentrations of 0.5 to 0.75% increased sperm motility and viability and protected sperm membrane integrity during short-term storage.

As an endogenous antioxidant in animals, glutathione (GSH) can significantly improve the quality of thawed semen when it is added to the used sperm diluent. Makarevich et al. (34) investigated the effect of GSH (at doses of 0. 5 mM, 1. 5 mM and 5 mM) on motility and viability of ram semen after semen storage under cooling conditions for several days. The results showed that the inclusion of GSH in the extender could maintain the quality of sperm stored at 5°C for at least 48 hours. The authors highlighted the need for further studies of different concentrations of GSH to draw a firm conclusion about its effect on spermatozoa functional activity. The majority of studies reported either no effects or detrimental effects of high concentrations (50-400mM) of GSH, while most positive effects on ram and bull sperms were recorded when medium (5-10 mM) or low (0.2-5 mM) concentrations were used (67). Curcumin and methionine are other antioxidants containing in the semen extenders (68-70). Curcumin is a natural antioxidant shown as an antiapoptotic and cryoprotective agent with anti-inflammatory, antitoxic, and anticancer effects (71-74). Methionine may act as a precursor amino acid for glutathione. It protects cells from oxidative damage and has a vital role in detoxification (75). Additionally, methionine plays an important role in the antioxidant defence system by readily reacting with oxidants to form methionine sulfoxide (76). Ellagic acid has potent antioxidant activity, radical scavenging capacity as well as chemopreventive and antiapoptotic characteristics (77, 78). Omur et al. (79) studied the protective effect of methionine, curcumin and ellagic acid over sperm motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in frozen

semen samples from Merino rams. They found that membrane integrity was preserved by 1 mM methionine (64. 2 ± 2. 3), 1 and 2 mM curcumin (60. 1 ± 2. 4 and 61. 6 ± 1. 1), and 1 and 2 mM ellagic acid (63. 0 ± 1. 7 and 61. 6 ± 3. 7). Positive effects in terms of acrosome status and motility compared to controls were observed for all antioxidant groups. The authors concluded that different concentrations of curcumin, methionine and ellagic acid had significantly different efficacy in the spermatozoa preservation. Melatonin (MLT, N-acetyl-5-methoxytryptamine) is a potent non-enzymatic antioxidant synthesized and secreted by the pineal gland of all mammalian species (80, 81). Recently, the antioxidant capacity of MLT and its metabolite derivatives has been established to act synergistically with other classical antioxidants to protect organisms from oxidative stress (82, 83). Kumar et al. (84) reported for effect of different concentrations (0.5mM, 1mM, 2mM) melatonin on the storage of cooled ram semen. The semen quality and the oxidative stress indicators (Total Antioxidant Activity and Malondialdehyde) in the samples with 0.5 mM and especially 1 mM MLT showed significant improvement. Pool et al. (85) applied melatonin treatment for improvement of the sperm function or fertility in Merino rams during anoestral season. This treatment improved some aspects of sperm function, but no change in fertilization rates was recorded. The supplementation of superoxide dismutase and catalase to Tris diluents also can preserve ram semen. It results in increased motility, preservation of acrosome integrity, and improved fertilization ability (86). In addition to the incorporation of various antioxidant and antioxidant compounds in semen extenders, the literature also addresses the effect of sources of long-chain polyunsaturated fatty acids, mainly omega n-3 included in the diet of male animals on sperm quality (87).

CONCLUSION

The high sensitivity of sheep spermatozoa to various exogenous and endogenous stress factors and the decreased fertility rate of chilled and especially frozen semen, trigger the need for further studies. The complex biochemical processes occurring in the male gametes during *in vitro* studies (accumulation of free radicals with consequent damage to spermatozoa integrity and function) have to be investigated in respect of improving sperm quality and fertilization capacity after long-term semen storage. Detailed investigations on the intimate mechanisms of oxidative stress attributed to its influence at the cellular level will give additional information about the possibilities about reduction of the negative effects on the spermatozoa. Different types and concentrations of enzymatic and non-enzymatic antioxidants can be used for the improvement of the semen extender quality. All the above mentioned may be a key to improving the current methods and developing new tools for the effective utilization of ram semen.

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