The Protective Effect of Antioxidants on Liquid and Frozen Stored Ram Semen – Review

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Abstract
This systematic review is focusing on the current literature in order to give an overview of the protective role of antioxidants in ram semen preservation. Throughout the sperm conservation process the unsaturated fatty acids of the spermatozoa membrane binds oxygen and evolves numerous peroxide bonds. The lipid peroxidation leads to unbalanced oxidative stress that causes different impairments of sperm cells, and acrosome loss. „Cold shock" also induces caspase cascade involved in apoptosis, DNA fragmentation and in overall it has a detrimental effect on the fertilizing capacity of spermatozoa. Nowadays the cryopreservation of semen is considered as a routine procedure in cattle. Despite the various advantages of the method, the recovery rate of live and intact spermatozoa still remains low in boar, dog and ram samples. Previously several studies highlighted that the addition of antioxidants could improve the survival and motility rates, because antioxidants acted as free radical scavengers and protected spermatozoa against reactive oxygen species (ROS). Enzymatic antioxidants as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and non-enzymatic antioxidant molecules like tocopherol, ascorbic acid, pyruvate, resveratrol have a protective effect against membrane damage that occurs during semen preservation process.

Keywords: Antioxidant, curcumin, ram, semen, sheep

1. The effect of cold shock and oxidative stress on ram semen

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses in the body. The cause of oxidative stress is considered as a major conducive factor to male infertility and decreased semen quality during the preservation process [1]. All cellular components (e.g. nucleic acids, lipids and sugars) are possible targets of oxidative stress; moreover the consequence of excessive ROS production leads to lipid peroxidation, decreased motility and viability and increased mid-piece sperm morphological defects. In the past decades many experiments have been conducted to clarify the effects of oxidative stress on different cell types; however the effect of free radical generation on spermatozoa is still unexplored [2]. Ram sperm cells are vulnerable to free radical attack, since are rich in polyunsaturated fatty acids (PUFA), therefore ROS can combine readily with them, directly causing lipid peroxidation (LPO). The sensibility of PUFAs to free radical attack arises from the fact that the carbon hydrogen dissociation energies are the lowest at the bisallic methylene position. The emerging carbon-centered lipid radicals may be combined with oxygen to produce peroxyl (ROON) and alkyl (RON) radicals, extracting hydrogen atoms from adjacent carbons, resulting in lipid radicals

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maintaining the peroxidation chain reaction [3]. Lipid peroxidation chain reaction has a harmful effect on both the movement and the physical attribution of the membrane surface structure of the cells that may lead to diminished membrane fluidity. These fundamental membrane changes in the spermatozoa deprive the competence of these cells for fertilizing the egg [4]. *Figure 1* presents the effect of oxidative stress on spermatozoa. Silva et al., 2006 [5] described several methods used to assess semen deterioration caused by ROS species. To assess plasma membrane integrity membrane-impermeable dyes are used e.g. Hoechst 33258; YoPro-1; propidium iodide or ethidium homodimer; ToPro-3 and TOTO. Sperm cells are considered to be alive if they exclude the mentioned fluorescent dyes [5, 6]. Acrosome reaction is mainly detected by the use of lecithin conjugates bind to carbonhydrate moieties of glycoproteins localized in the acrosome. For acrosome injury recognition mainly fluorescent probes are used as PNA-FITC, PNA-TRITC and PNA-RPE [5, 7]. Organelle-specific dyes e.g. Mitotracker Deep Red TM, Mitotracker Red TM, Mitotracker Orange TM and Mitotracker Green TM are used to label the respirating mitochondria. Under oxidative respiration Mitotracker Orange CM-H_2TMROS and Mitotracker X-Rosamine CM-H_2XROS are widely used to distinguish spermatozoa with deteriorated mitochondria from aerobically accomplished sperm cells. Lipid peroxidation and the distortion in the lateral and bilayer organization of lipids as well can be localized and quantified with C_{11}BODIPY fluorescent probe which is an analog for unsaturated fatty acids, the main targets for ROS. Sperm DNA integrity can be measured at three different levels: the assessment of DNA condensation status using single-cell DNA gel electrophoresis assay (COMET); transmission and electron microscopy (TEM); or Chromomycin A3 can be used to follow the last compaction steps of DNA to protamines. Furthermore DNA nicks and breaks can be detected by the use of Acridine Orange fluorescent stain or by using TUNEL when enzymatic incorporation is allowed of fluorescent nucleotide analogs by a terminal nucleotide transferase into single stranded DNA areas [8].

Despite the harmful effect of ROS on sperm cells ROS production may act as an important mediator of sperm function. Controlled generation of ROS is necessary for the development of capacitation and hyperactivation that ensures the fertilization in the female reproductive tract, therefore a limited level of ROS species is adequate to achieve an appropriate sperm function [9]. Seminal plasma antioxidants [e.g. glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT)] as enzyme scavengers of ROS are able to degrade superoxide anion, hydrogen peroxide and lipid peroxides [10]. SOD and CAT are able to inactivate superoxide anion (O_2) and peroxide radicals (H_2O_2) by neutralizing them into water and oxygen. SOD is also responsible to maintain a balance between ROS generation and degradation [11]. Recent work has shown that the endogenous glutathione contents are the highest in mouse and bull spermatozoa, intermediate in human, ram and are low or undetectable in boar and rabbit semen [12]. Ram seminal plasma has a very effective antioxidant system in which the antioxidant level shows differences during the year. Seasonal changes in superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT) in seminal plasma (SP) of mature Rasa Aragonesa rams were determined. The semen collected in non-breeding season (March-September) had the highest antioxidant enzyme activity further in all year around SOD and CAT activities were higher in the first ejaculates compared to the second ones. However, GR and GPx activities changed throughout the year [13]. In order for the semen cooling and freezing process to be successful, it is necessary to know the effect of the antioxidants on sperm freezing and fertility results.

2. The liquid and frozen storage of ram semen

When the use of AI started to expand it also required the transport of ram semen to more distant farms, therefore semen storage technology was revolutionized to improve the storage of semen under artificial conditions. To ameliorate poor sperm survival and to be able to prolong sperm fertility for AI, researchers explored the liquid (unfrozen) and frozen storage of semen [14]. Cold-shock is a phenomenon that causes irreversible changes e.g. capacitation, apoptosis and DNA hypomethylation when semen is stored at low temperature. Fowl and human spermatozoa are classified as more cold-shock resistant than bull and ram spermatozoa nevertheless by using additives such as egg yolk, milk, TRIS, glycerol,

antioxidants during slow cooling the cold shock effect could be minimized [15]. The irreversible effect of cold shock occurs at rapid cooling of semen from 30 °C to various temperatures above 0 degrees.

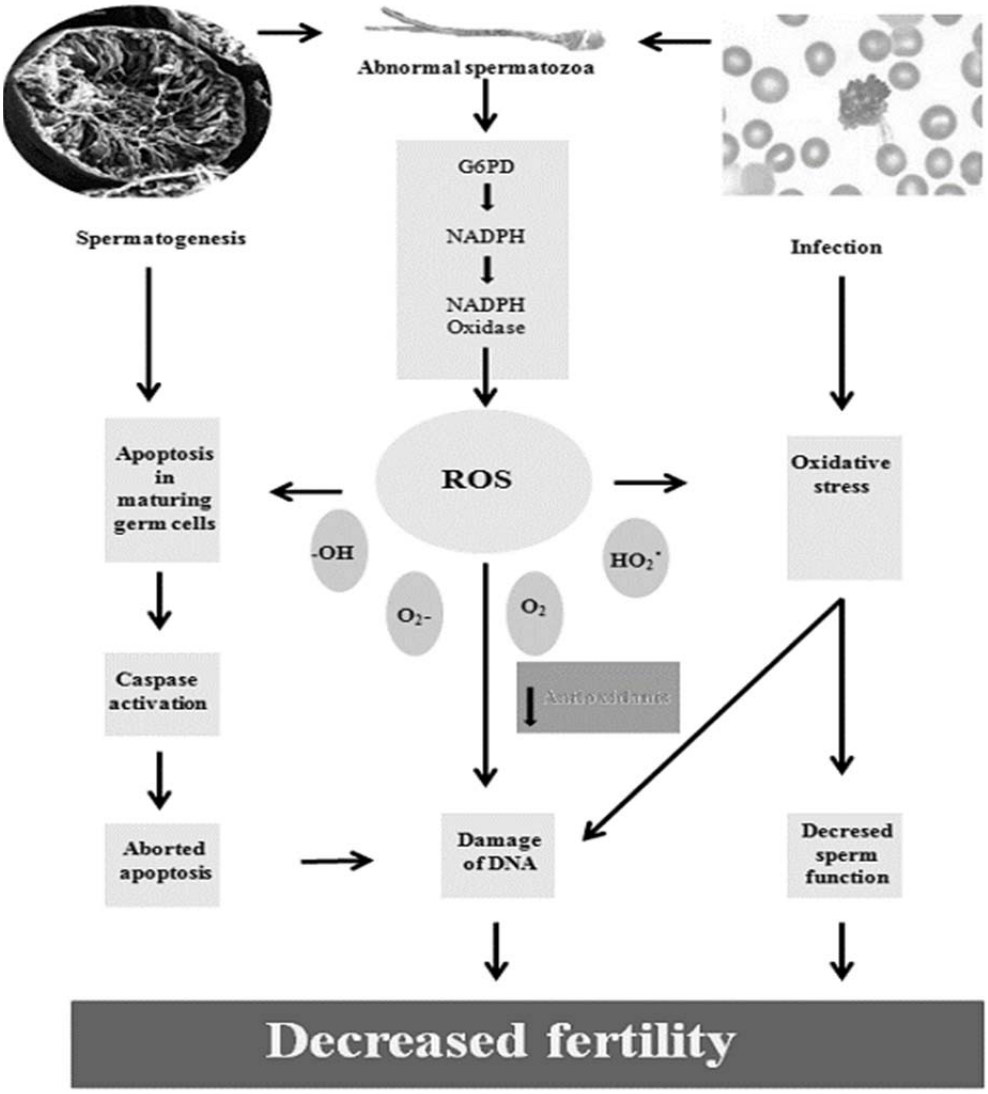

Figure 1. The effect of oxidative stress on semen

Rapid cooling of extended semen to 10, 5 or 0 degrees is associated with decrease in the progressive motility and intact acrosome rates [16; 17]. By the addition of seminal plasma proteins higher viability could be achieved in ram spermatozoa samples freed from seminal plasma [18]. Rovegno et al., 2013 [19] also described that the addition of seminal plasma to post thawed ram semen is capable of improving sperm resistance to thermal shock, reverting cryocapacitation and helping sperm survival. Furthermore seminal plasma proteins are able to repair the damaged plasma membrane and have a protective effect on plasma membrane integrity and potential as well. In addition seminal plasma proteins play a key role by the inhibition of capacitation and the possibility is proposed, that seminal plasma proteins might interfere with the extrinsic and intrinsic apoptotic pathways [20]. The liquid storage of ram semen is successfully used to depress sperm metabolism and to use the semen for AI up to two days after semen collection. Unlike bovine, ram semen has a reduced fertility after the cryopreservation process. Ram semen freezing leads to the decrease of motility by minimum 20%, therefore cryopreservation

methods still need to be improved [21]. To reduce sperm metabolism to prolong viability short term conservation between 10-15 °C is commonly used. Liquid storage at 0-5 °C maintains the viability and fertility of ram spermatozoa for a longer time, when cells preserved up to 10 days at 5 °C lead to high fertilization rates by using in vitro fertilization (IVF) (Maxwell and Salamon, 1993 [22]; Menchaca et al., 2005 [23] used chilled ram semen (5 °C) for AI. Semen was diluted with TRIS based extender and was used after 24h, 12h and soon after collection, and the conception rates were 34.5%, 42.7% and 50.4%. These results also confirm that by using fresh, diluted ram semen for AI still better fertility results can be achieved. For freezing and thawing semen various methods have been described. The methods and diluents used for bull semen freezing were mainly adopted to ram, then alterations were made to prolong viability and to accomplish better fertility results [24; 25]. Semen freezing and thawing causes functional and ultrastructural damages of spermatozoa that lead to reduction in motility, impaired transport and the loss of fertilizing capacity [26]. Although many years of research has established a variety of methods in order to improve the process, fertility is generally lower also when liquid stored ram semen is used, nevertheless using antioxidants in the extenders could be a promising solution to prevent or reserve detrimental membrane changes during the chilling, freezing and thawing process [27]. The main goal is to find an antioxidant, or combination of different ones, that could prolong sperm viability.

3. The effect of enzymatic antioxidants

Many studies have already examined the effect of enzymatic antioxidants on semen storage. Seminal plasma and spermatozoa themselves have endogenous antioxidants in order to protect spermatozoa from ROS. Spermatozoa predominantly possess three main antioxidants as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase/glutathione reductase (GPx). Low Total Antioxidant Capacity (TAC) in seminal plasma has a key role in infertility, TAC is the sum of enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic (e.g. ascorbate, urate, tocopherol, pyruvate, glutathione, taurine and hypotaurine) antioxidants. Giulini et al., (2009) [28]; Camara et al., (2011) [29] supplemented diluted ram semen with various antioxidants e.g. glutathione, superoxide dismutase and catalase in different concentrations, but the additives did not increase the total antioxidant capacity of semen, nor did enhance the quality of post-thaw samples. In contrast the addition of SOD (100 U⁄ml) and GSH (2 and 5 mM) can preserve the acrosome integrity of frozen ram spermatozoa, while the addition of 100 U⁄ml SOD to Tris egg-yolk extender may protect the cell membranes after thawing. Silva and Soares (2011) [30]; Camara et al., (2011) [31] and Foruzanfar et al., (2013) [32] used MnTBAP, a superoxide-dismutase mimetic and also a cell permeable antioxidant, investigating membrane integrity, capacitation status and in vitro fertilization ability of frozen-thawed ram semen. Adding 100 and 150 μM of MnTBAP to Bioxcell extender had a beneficial effect by increased number of non-capacitated cells compared to the non-treated group and the harmful effects of cryopreservation on membrane integrity were decreased, as well. For IVF 100 mM MnTBAP concentration appeared to be the best resulting in the lowest rate of non-capacitated spermatozoa. Maia et al., (2010) [33] evaluated the effect of antioxidant additives (Trolox, catalase) by using them in Tris-egg yolk freezing extender to quantify lipid peroxidation and hydrogen peroxide generation after thawing. The final results provided evidence that both treatments significantly reduced lipid-peroxidation and hydrogen peroxide concentration in the samples ($P<0.05$) and both antioxidants can be able to control the effect of oxidative stress in ram semen cryopreservation process.

4. The effect of non-enzymatic antioxidants

These antioxidants in semen include minerals, vitamins, amino acids and protein compounds (e.g. zinc, tocopherol, albumin, glutathione, taurine, hypotaurine, carnitine, carotenoids, urate and prostasomes) [34]. Uysal (2007) [35] investigated the effect of the addition of antioxidants as oxidized glutathione (GSSG), bovine serum albumin (BSA), cysteine and lycopene to freezing media on the post-thawing sperm characteristics (motility, morphology, acrosome integrity, viability and membrane integrity) by using Tris-based extender. Result provided that GSSG at 5 mM had a significant ($P < 0.001$) effect in maintaining post-thaw sperm motility, morphology, acrosome integrity and

viability (membrane integrity). Cysteine at 10 mM had a significant ($P < 0.001$) effect on maintaining post-thawing sperm characteristics while lycopene at 800 μg displayed significantly ($P< 0.001$) the best post-thawing spermatological indicators when compared to the other groups. Spalekova and Makarevich (2012) [36] studied the effect of glutathione on selected motion parameters on cool (4 °C) stored ram semen, diluted with Triladyl extender. Motion parameters were measured by CASA system. After 48h storage progressive motility remained higher in the glutathione supplemented sample, compared to the control group. The addition of catalase (100 and 200 U/mL) was also effective to reduce the deteriorative effects of cooling on total motility when ram semen was maintained at 5 °C for 24h in Tris-egg yolk extender. Bucak et al., (2007) [37] studied the effect of trehalose (50; 100 mM), taurine (20; 50 mM), cysteamine (5; 10 mM) and hyaluron acid (0.5; 1 mg/ml) by using them as ROS scavengers in Tris-based extender for frozen ram semen. Tris-base extenders supplemented with 50 mM trehalose, 25 mM taurine, and 5 and 10 mM cysteamine led to higher percentages of post-thaw motility, in comparison to the control group ($P<0.01$). Furthermore no significant differences were observed in the percentages of acrosome and total abnormalities, and the results of hypoosmotic swelling test. Bucak et al., (2012) [38] determined the effects of curcumin and dithioerythritol added to bull semen. Following the freezing/thawing process sperm parameters, lipid peroxidation, total glutathione and antioxidant potential levels of bull spermatozoa were quantified. By using 0.5 mM dose of curcumin supplementation lower percentage of total abnormality (20.40±2.36%) was achieved compared to the control (30.60±1.47%, $P<0.05$). Curcumin and dithioerythritol at 0.5 mM provided a greater protective effect on the membrane functional integrity (54.40±2.09% and 50.00 ±2.68%) in comparison to the control (37.20±1.77%, $P<0.001$). Nevertheless supplementation with these antioxidants did not significantly affect the lipid peroxidation and antioxidant potential levels.

Sarlós et al., (2002) [39] incubated Tris-egg yolk diluted ram semen at 37 °C for 30, 60 and 120 min. and 24-h at 5 °C in the presence of alpha-tocopherol acetate (E), glutathione peroxidase (GP), Aromex (AR), resveratrol (R), resveratrol+vitamin E (RE), resveratrol+Aromex (RAR) and resveratrol+glutathione peroxidase (RGP). The peroxidation in the samples was evaluated by the analysis of malondialdehyde (MDA). Following 30-, 60- and 120-min storage the concentration of MDA in control and R-treated samples was 25.89, 36.91, 49.57 and 3.69, 3.74, 3.74 nmol/10^9 spermatozoa, respectively. Furthermore significantly more motile sperm cells were observed in the treated group as compared to the control; therefore these results indicate that resveratrol treatment can improve the efficiency of ram semen preservation. In agreement with the mentioned results Silva et al., (2012) [40] also concluded that the addition of 5 to 20 μg/mL of resveratrol or quercetin to the Tris-egg yolk-glycerol extender improved the mitochondrial membrane potential in frozen thawed ram semen. The effect of various levels of vitamin B_{12} supplementation in Tris extender was examined by Hamedani et al., (2013) [41] on ram semen. B_{12} supplementation significantly improved viability, motility, progressive motility and decreased morphological defects. The optimum concentration to achieve the best results after 7 days of frozen storage was 2 mg/mL of B_{12} in Tris-based extender. Pivko et al., (2009) [42] carried out a study by using caffeine to examine the effect on the membranes and acrosomal status of ram spermatozoa. Caffeine increased sperm motility, but caused damages in sperm head membranes and induced pseudoacrosomal reaction after dilution.

5. Conclusions

Antioxidants, in general, are scavengers which restrain the formation of ROS species. Enzymatic and non-enzymatic antioxidants have the ability to attack ROS and LPO to protect sperm cells from membrane damage. A variety of defense mechanisms encompassing antioxidant enzymes, e.g. superoxide dismutase, catalase, glutathione peroxidase and reductase are involved in biological systems. Recent studies described that a balance between the benefits and risks from ROS and antioxidants appears to be essential for the normal functioning of spermatozoa. Seminal plasma also seems to be one of the most powerful antioxidant fluids in the organism, nevertheless supplementation of the extenders with antioxidants is recommended to facilitate the

enhancement of sperm cryopreservation techniques.

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