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Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation

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ABSTRACT

Ram sperm are subjected to extreme oxidative stress during their preservation at −196 °C resulting in reduced quality at post thaw. Therefore, the main objective of this study was to evaluate the effect of antioxidants taurine, quercetin and reduced glutathione on the post thaw quality of crossbred ram sperm. A total of twenty four ejaculates from six crossbred rams were collected and extended with tris-based extender with no antioxidant (Control), with taurine (40 mM), quercetin (5 μg/ml) and reduced glutathione (5 mM). The post thaw sperm quality was determined by percent sperm motility, live sperm count, intact acrosome and hypo-osmotic swelling test (HOST) reacted spermatozoa and lipid peroxidation was measured in terms of malondialdehyde (MDA) level both in seminal plasma and sperm cell. At post thaw, percent sperm motility and live sperm count were significantly ($p < 0.05$) higher for taurine than control and reduced glutathione but did not differ significantly ($p > 0.05$) from quercetin. The percent HOST reacted spermatozoa were significantly higher for taurine than control, quercetin and reduced glutathione. Seminal plasma MDA level was significantly ($p < 0.05$) lower for taurine than control and non-significantly lower than quercetin and reduced glutathione. However, spermatic MDA level did not differ significantly ($p > 0.05$) among the control and antioxidants. In conclusion, taurine at 40 mM reduced lipid peroxidation and improved post thaw sperm quality of cryopreserved crossbred ram semen. Further, transportation time of semen samples in an ice chest at 4–5 °C may be included as a part of equilibration period, when collection shed and frozen semen unit are located at a distance.

1. Introduction

Artificial insemination (AI) with frozen semen in sheep can result in substantial improvement in inferior genetics, control of venereal diseases [26] and even individual farmers may be greatly benefitted [10,30]. With the advent of artificial insemination technology, there has been a tremendous genetic improvement in cattle but similar results have not been translated in sheep. The reason being either poor post thaw ram semen quality or failure to penetrate the AI gun through the cervix owing to its tortuous character to deposit semen either at the deep cervix or in the uterus for better pregnancy rate [16,30,47]. Deposition of frozen semen near the external os cervix has resulted in poor conception rate [28,10]. Many unsuccessful attempts have been made to dilate the ovine cervix for penetration and deposition of frozen semen in the uterus. An alternative to achieve better pregnancy rate is to increase post thaw semen quality [38,2], so that even if semen is deposited near the os cervix, a good number sperm could gain entry via the cervix and an optimum sperm reservoir gets established at the caudal isthmus for successful fertilization. Further, the improvement of AI success via the transcervical route has a positive ethical aspect as well as a practical advantage. The alternative surgical method (Laproscopic), where semen is injected directly into the uterine horns of sheep and females are subjected to surgery under local anaesthesia. Therefore, improvement in the technology for freezing ram semen might be able to reduce this practice.

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Sperm cryopreservation has emerged as an important tool in biology to disseminate superior germplasm and maintain genetic diversity [43]. However, the process of cryopreservation is a damaging phenomenon [4] resulting in deterioration of sperm quality due to excess production of oxygen free radicals during sperm freeze-thawing cycles [39,51]. A basic cause to this damage is the presence of high content of polyunsaturated fatty acids (PUFA) in the plasma membrane of spermatozoa [24,50,56]. These PUFA particularly of dead spermatozoa [41] bind with oxygen resulting in the production of high level of reactive oxygen species (ROS) [34,21]. The ram semen is endowed with a robust antioxidant enzyme system in the form of reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD) to scavenge ROS [1,22]. Despite this, the endogenous antioxidant system is not capable to scavenge the excess ROS generated mostly from dead sperm leading to an imbalance between the production and elimination of ROS resulting in oxidative stress [20]. This leads to loss of structural and functional integrity of membranes, increasing membrane permeability, DNA structural damage and cell death [1,22,25]. To this, Inclusion of a suitable antioxidant in semen extender could prove beneficial in reducing the oxidative stress thereby protecting spermatozoa during freezing and thawing [4,10,15,45]. Taurine (2-amino-ethane-sulfonic acid) a free β-amino acid, is neither a classical scavenger nor a regulator of the antioxidative defences, however it regulates mitochondrial protein synthesis protecting the mitochondria against excessive superoxide generation [27]. Taurine has been found to protect ram sperm membrane against lipid peroxidation and loss of sperm motility [57,45]. Quercetin, a flavonoid polyphenol, is the most potent scavenger of reactive oxygen species [18,23] and has been shown to improve post thaw sperm quality in ram [52] and stallion [51]. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine), an enzymatic antioxidant, plays an important role in the intracellular defence mechanism against oxidative stress [10]. There is a meagre evidence and ambiguity in the results obtained by different workers on addition of antioxidants like taurine, quercetin and reduced glutathione for improving post thaw sperm quality in crossbred ram. Therefore, the present study was designed to investigate the effect of antioxidants on lipid peroxidation (LPO) and quality during cryopreservation of cross bred ram semen.

2. Materials and methods

2.1. Chemicals

The chemicals used in the study were procured from Hi-Media laboratories Pvt Ltd (India) and Merck specialities Pvt Ltd (India).

2.2. Selection of animals

Six healthy crossbred rams between the age group of 4–6 years, maintained at Mountain Research Centre for Sheep and Goats (MRCSG), Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, located at the outskirts of Srinagar city, Kashmir province, India (34°08′N 74°28′E) were randomly selected for this study. The selected rams were kept under similar managemental conditions during the entire study.

2.3. Collection of semen, evaluation and cryopreservation

A total of 24 ejaculates were collected from selected rams three times a week during the breeding season (autumn) by artificial vagina (AV) method in the morning. Prior to collection, the preputial hair was clipped and thoroughly washed with normal saline to minimise the chances of contamination. The ejaculate from each ram was grossly evaluated for colour, volume, consistency and presence of any foreign body. Ejaculates with normal colour, volume (>0.5 ml), consistency and without any foreign body were initially diluted with buffer in the ratio of 1:1 and then evaluated for initial motility. A minimum of four ejaculates with initial motility greater than or equal to 70% were pooled together (4 ejaculates per pool and total 6 pools) to avoid individual variations and a representative sample was thus obtained . A portion of pooled sample was extended with tris extender and maintained at 30–34 °C in a separate tube for pre freeze sperm quality evaluation. The remaining pooled semen sample was divided into four aliquots designated as control, taurine, quercetin and reduced glutathione and were subsequently extended in tris extender without (Tris citric acid buffer 73 ml; Fructose 1.25 g; Egg yolk 20 ml; glycerol 7 ml; Penicillin G sodium 80,000 IU; Streptomycin 100 mg) or with different antioxidants (Tris extender + Taurine 40 mM; Tris extender + Quercetin 0.000014 mM or 5 μg/ml; Tris extender + Reduced glutathione 5 mM). Tris citric acid buffer was prepared by adding distilled water to 3.028 g of tris (Hydroxy methyl amino methane) and 1.7 g of citric acid monohydrate up to 100 ml. The final sperm concentration in the extended samples was adjusted at 600 million/ml. The extended semen samples were immediately placed in an ice chest and transported to the frozen semen station for further processing. The distance from collection shed and frozen semen station is nearly 40 km and it took 3 h from initial processing at the faculty laboratory to reach at frozen semen station. Therefore, the time from when samples were placed in an ice chest to when temperature of 4–5 °C was attained was about 45–50 min (cooling) and rest of the time was included in equilibration period of 4 h. Upon reaching the frozen semen station the time left for equilibration was completed in cold handling cabinet. The equilibrated semen samples were then aspirated into French mini straws (0.25 ml) with an automatic filling and sealing machine (IMV Technologies, France). The final sperm concentration remained 150 million/straw. After this, the straws were subjected to rapid vapour freezing in a Biological Programmable Freezer (IMV Technologies, France). The temperature was initially reduced @ -5 °C min^{-1} from +4 °C to −10 °C, then @ −40 °C min^{-1} from −10 °C to −100 °C and finally @ −20 °C min^{-1} from −100 °C to −140 °C. The pressure inside the Biological Programmable Freezer (IMV Technologies, France) was maintained at 3 Bars. After vapour freezing, the straws were finally put in pre-cooled goblets for deep freezing in liquid nitrogen.

2.4. Pre freeze sperm quality evaluation

2.4.1. Sperm motility and live sperm count

For sperm motility, 100 μl of semen sample was placed on a clean grease free slide maintained at 37 °C. A cover slip was then put on the sample and observed at 400× magnification of phase contrast microscope (Nikon Eclipse E200, Japan). Spermatozoa moving in forward direction were estimated and expressed in percentage. The semen samples were analysed by three researchers in three different fields and an average value of the three readings was considered as a final value of the progressive sperm motility. The live sperm count was determined by using eosin-nigrosin stain. The stain was prepared by dissolving 5 g of Eosin-Y (water soluble) and 10 g of Nigrosin separately in 100 ml of 2.9% sodium citrate solution. The mixture was boiled for 15 min. The volume which evaporated during boiling was replaced with the buffer. The final stain was prepared by mixing 5% Eosin-Y solution and 10% Nigrosin solution in a ratio of 1:4 in a container. The mixture was then shaken thoroughly and filtered through Whatmans filter paper. A drop of semen sample was mixed with three drops of stain and the semen-stain mixture was allowed to rest for 2 min. After 2 min, a