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Effect of L-cysteine Supplementation on Cryopreservation of Black Bengal Buck Semen

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ABSTRACT

Background: Black Bengal goat is one among the valuable goat breeds of India. Semen preservation and artificial insemination are practical technologies for conservation and improving livestock germplasm. Various biochemical changes that occur during cryopreservation of semen, results in loss of sperm cell structure and their functions. Inclusion of antioxidants to the semen extender has been reported to have positive effects on sperm recovery in various livestock species. The present experiment was carried out to evaluate the effect of antioxidant L-cysteine on post freeze thaw sperm characters, lipid peroxide level and superoxide dismutase (SOD) activity during cryo-preservation of Black Bengal buck semen.

Methods: Twenty numbers of semen ejaculates were collected from Black Bengal bucks, antioxidant L cysteine was added to the semen extender @ 0, 1 and 2 mM in control group (CC), treatment group 1 (CT_1) and treatment group 2 (CT_2), respectively. Semen samples were frozen in liquid nitrogen and measured post freeze-thaw *in vitro* sperm characters, malondialdehyde (MDA) concentration and SOD activity.

Result: Though there was no significant improvement in post thaw sperm motility, functional membrane integrity and viability count, supplementation of L-cysteine significantly improved the percentage of sperm cells with intact acrosome and significantly ($P<0.05$) reduced the level of malondialdehyde. Post thaw SOD activity was found significantly lower in the antioxidant treated groups than the control. It is concluded that supplementation of L cysteine is effective in controlling lipid peroxidation and conserving *in vitro* sperm characters during freezing of Black Bengal buck semen.

Key words: Antioxidants, Black Bengal goat, Buck semen, *In vitro* characters.

INTRODUCTION

The highly prolific breed of goat, Black Bengal (*Capra hircus bengalensis*) is distributed throughout Eastern part of India and Bangladesh. Black Bengal goats are known for their quality cheavon with characteristic low intramuscular fat (Islam *et al.*, 1991). The high demand of the buck meat resulted in reduction of male population compared to females and the ratio of male and female has reduced to 1.13: 88.7 (Nandi *et al.*, 2011). In this circumstance, farmers are slowly moving towards adoption of artificial insemination. To get better conception rate, semen straws should contain sufficient numbers of progressive motile and intact sperm cells. Damages to spermatozoa all through the cryopreservation and thawing occur frequently due to oxidative stress as an end result of production of free radicals or excessive Reactive Oxygen Species (ROS). Oxidative sperm damages lead of DNA fragmentation, altered mobility and decreased potential of the spermatic membrane to fuse with oocytes. Although seminal plasma of mammalian semen contain numerous antioxidants such as glutathione peroxidase, catalase, superoxide dismutase and free radical scavengers such as albumin, vitamins C and E, taurine, *etc.* (Zini *et al.*, 2002), the levels of seminal antioxidants are reduced during the dilution and freezing of the semen sample. Lack of sufficient antioxidants in semen extender has effects on the sperm survivability during the process of cryopreservation and thawing. External administration of antioxidants can reduce this oxidative stress and sustain the activity of the spermatozoa. L-cysteine is an amino acid having thiol (-SH) group and is considered as a precursor of intracellular glutathione (Grunewald *et al.*, 2005). Aitken *et al.* (1997) found that it can easily penetrate through cell plasma membrane, improving the intracellular biosynthesis of glutathione and thereby protecting the cell membrane from oxidative damages. Thus, here we have analysed the effect of antioxidant L-cysteine on cryopreservation of Black Bengal buck semen.

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MATERIALS AND METHODS

The present study was carried out at ICAR-National Dairy Research Institute (NDRI), Eastern Regional Station, Kalyani, West Bengal, India, during 2019 to 2020. This experiment was approved by the Institute Research Council of ICAR National Dairy Research Institute, Karnal, India. Black Bengal bucks (n=8) of 1.5 to 3.5 years of age were used for donating semen for the study. The fresh semen ejaculates were collected once a week with artificial vagina. Semen samples with sperm cell concentration $\geq 2500 \times 10^6$/ml, mass activity $\geq$ 3 + and individual motility more than 70% were selected for cryopreservation. The basic extender was prepared by mixing 300 mM Tris, 28 mM glucose, 95 mM citric acid, egg yolk 20% (v/v) and 500 µg/ml gentamicin in distilled water respectively (Konyak *et al*., 2018). A single step addition of cryoprotectant glycerol (5% v/v) into the extender was performed. To evaluate the effect of L- cysteine on cryopreservation, it was added to the semen extender @ 0 mM in control group (CC), @ 1 mM in treatment group 1 (CT_1) and @ 2 mM in treatment group 2 (CT_2). The semen was diluted in such a way that the final extended semen contained the sperm cell concentration of at least 300×10^6 sperm cells per ml. In this experiment, 20 numbers of semen ejaculates were used to study the effect of cysteine on cryopreservation. The extended semen samples were filled manually in 0.25 ml French mini straws and sealed. The straws were equilibrated for 3 hours at refrigeration temperature and frozen in liquid nitrogen by vapour freezing method (Karunakaran *et al*., 2019). The semen straws were evaluated for post thaw *in vitro* characters such as sperm motility, functional membrane integrity, viability, acrosome membrane integrity, concentration of lipid peroxide compound malondialdehyde (MDA) and level of superoxide dismutase activity (SOD). Sperm motility was assessed by examining a drop (10 µl) of thawed semen sample by light microscopy. Sperm cell viability was assessed by eosin-nigrosin staining. Hypoosmotic swelling test with eosin staining (HE-test) was used to evaluate functional membrane integrity. A total of 200 sperm from each replication were counted for coiled and uncoiled tails under bright field microscope at 400 magnifications (Konyak *et al*., 2018). The acrosome integrity was analysed by Giemsa stain. Lipid peroxidation level of spermatozoa in semen sample was estimated by measuring the malondialdehyde (MDA), using thiobarbituric acid (Karunakaran and Devanathan, 2016). Activity of superoxide dismutase (SOD) in the frozen thawed semen samples were estimated using commercially available kits as per the instructions of the manufacturer (Immuotag SOD assay kit, catalog # ITFA1010).

Statistical analysis

All percentage data were subjected to arc sine transformation to overcome the scale effects and for normalization of data and one-way ANOVA was applied. The means for arc sine percentile values of all seminal traits were back- transformed and presented as mean percentage. Differences were considered significant at P<0.05.

RESULTS AND DISCUSSION

In our study, the mean values of 77.65±1.18% progressive forward motility, 58.40±1.43% functional membrane integrity, 58.50±1.12% viable cells and 65.05±1.63% acrosome intact sperm cells were recorded in the fresh semen samples of Black Bengal bucks immediately after dilution with semen extender. The mean values of the *in vitro* sperm characters in post thawed semen following supplementation with L-cysteine as antioxidant @ 0 mM (control), 1 mM (CT_1 group), 2 mM (CT_2 group) are presented in Table 1.

Supplementation of L-cysteine did not have any significant effect on the post thaw sperm motility and functional membrane integrity during cryopreservation of buck semen. Similar to current observation, Tuncer *et al.* (2010) found that the addition of 10 mM cysteine did not exhibit significant cryo-protective effect on the *in vitro* sperm parameters such as post-thaw motility and morphology. In contrary, Ogretmen *et al*. (2015) reported that supplementation of cysteine @ 20 mM in the media yielded significantly higher post thaw motility (76.00±1.00%), fertilization rates (97.00±1.73%), hatching rates and decreased DNA damage during cryopreservation of semen in common carp (*Cyprinus carpio*). Inclusion of 5 and 7.5 mM cysteine in semen extender significantly (p<0.05) increased post thaw sperm motility and plasma membrane integrity with significant reduction in intracellular ROS when compared with control groups during cryopreservation of buffalo bull semen was reported by Topraggaleh *et al.* (2014). Dolti *et al.* (2016) observed the post-thawed sperm motility was significantly higher in the extender added with 4 mM cysteine and 50 mM trehalose during cryopreservation of ram semen. Further, they reported that the spermatozoa

Table 1: *In vitro* sperm characters (Mean±SEM) in post thawed semen of Black Bengal bucks supplemented with L-cysteine.

Parameters	Control (CC)	L-cysteine 1 mM (CT_1)	L-cysteine 2 mM (CT_2)
Progressive forward motility (%)	45.17±3.14	50.17±3.1	51.61±2.77
Functional membrane integrity (%)	35.35±1.77	37.15±1.77	37.60±2.02
Sperm viability (%)	37.65±1.65	39.45±1.66	39.6±1.80
Acrosome integrity (%)	52.35±4.32^{a}	53±4.23^{ab}	55.45±4.55^{b}
MDA (µmol/ml)	2.384±0.12^{aA}	1.625±0.254^{bB}	0.885±0.304^{cC}
SOD (U/mg of protein)	0.381±0.031^{aA}	0.223±0.01^{bB}	0.115±0.004^{cC}

Rows with different superscripts $^{a, b, c / A, B, C}$ differ significantly @ P<0.05 and P<0.01, respectively (n=20).

motility decreased gradually in diluents containing cysteine and trehalose compared to control group. Ansari *et al.* (2016) reported that the percentage of sperm cells with intact plasma membrane was higher in extender supplemented with 0.5 mM and 1.0 mM cysteine than the untreated control during cryopreservation of buffalo bull semen. El-Sheshtawy *et al.* (2008) also reported a significantly higher percentage of sperm with intact plasma membrane after freeze-thawing with extender included with cysteine (5 mM) in Egyptian buffalo semen. Similarly, higher percentage of sperm cells with functional plasma membrane was observed in bull (Sariözkan *et al*., 2009) and ram (Uysal and Bucak, 2007) semen frozen-thawed in semen extender added with 2.0mM and 5.0 mM cysteine. The overall mean values of viable sperm cells observed were 37.65±1.65, 39.45±1.66 and 39.60±1.80% in control, CT_1 with 1 mM L-cysteine and CT_2 group supplemented with 2 mM L-cysteine, respectively. In the present study, supplementation of L-cysteine @ 1 and 2 mM resulted in a non-significant improvement in the percentage of viable sperm cells in post thawed buck semen. Ansari *et al*. (2016) attained significantly higher viable sperm cells in samples cryopreserved in tris-citric acid extender containing cysteine 0.5 mM and 1.0 mM compared with the control in buffalo bull semen. Similarly, Bucak *et al*. (2008) also reported improvement in % of viable sperm cells after addition of 5.0 mM cysteine in tris citric acid extender in ovine semen. Dolti *et al*. (2016) also reported that the extender containing of cysteine and trehalose had a significant ($p<0.05$) effect on sperm viability during preservation of ram semen. The overall mean values of acrosome intact sperm cells were, 52.35±4.32, 53.00±4.23 and 55.45±4.55% in control, CT_1 and CT_2 group, respectively. Non-significant difference was observed in mean acrosome intact sperm cells of the control and CT_1 groups and between the treatment groups but CT_2 group had significantly ($p<0.05$) more acrosome intact cells than the control group. Acrosome membrane integrity is essential for the sperm cells to undergo capacitation, acrosomal reaction and finally fertilization (Tartaglionea and Ritta, 2004). In the present study, buck semen samples supplemented with 2 mM L-cysteine had significantly more acrosome intact sperm cells than the untreated control group. Akin to this observation, Ansari *et al.* (2011 and 2016) recorded higher intact acrosome percentage in cryopreserved buffalo bull sperm due to addition of cysteine 1.0 mM. Further, Iqbal *et al*. (2016) also observed that post-thaw acrosome integrity were higher ($p<0.05$) with the addition of 2.0 mM L-cysteine in Nili-Ravi buffalo bull semen freezing. The mean values of MDA level in post thawed semen of Black Bengal buck were 2.384±0.12, 1.625±0.25 and 0.885±0.30 µmol/ml in control group without L-cysteine supplementation, CT_1 group with 1 mM L-cysteine and CT_2 group with 2mM L-cysteine respectively. Significant difference was observed between control and treatment groups and between treatment groups ($p<0.01$; $p<0.05$) in the mean concentration of lipid peroxide compound MDA. In this study, supplementation of L-cysteine @ 1 and 2 mM to the semen extender helped in controlling the development of lipid peroxide compound MDA during freezing of buck semen. Cysteine is a sulphur-containing amino acid, naturally found in seminal plasma and sperm nucleic acid, that acts as an antioxidant directly and/or indirectly through intracellular antioxidants that protect from ROS-mediated deleterious effects (Perumal *et al*., 2011; Topraggaleh *et al*., 2014). In contrary to the present findings, Tuncer *et al.* (2010) found that addition of 10 mM cysteine resulted in the highest level (4.99±0.44 nmol/mL) of MDA ($P<0.001$) when compared to the control (1.44±0.08). Similarly, Sariozkan *et al.* (2009) also reported that supplementation of cysteine @ 2 mM to semen extender for freezing of bull semen did not show effectiveness on the elimination of MDA formation when compared to controls. These contradictory results may be due to the differences of semen extender composition, animal species and cysteine concentration used in the study. The overall mean values of superoxide dismutase activity observed were 0.381±0.031, 0.223±0.01 and 0.115±0.004 (U/mg of protein) in control, CT_1 and CT_2 group, respectively. Significant difference was observed between control and treatment groups and between treatment groups ($p<0.01$; $p<0.05$). Similar to the current observation, Bucak *et al.* (2009) also reported that supplementation of the antioxidant cysteamine decreased SOD activity when compared to the controls ($p<0.001$) post freeze thawing of Angora goat semen. Coyan *et al.* (2011) also found that the supplementation of cysteine did not significantly affect activities of SOD and GPx in Merino ram semen.

CONCLUSION

In conclusion, present study showed that the supplementation of L-cysteine in the semen extender has a positive effect on post-thawed sperm survivability, acrosome integrity and controlling the development of lipid peroxide compound during cryopreservation of Black Bengal buck semen. Further studies with varying levels of L-cysteine and studies upon the conception rate and kidding size following artificial insemination with cysteine supplemented semen is required to confirm the beneficial effects of cysteine on cryopreservation of Black Bengal buck semen.

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Ethics approval

The work was carried out with the approval of Institute Research Council of ICAR-National Dairy Research Institute, Karnal, India and work did not require ethical committee approval.

Conflicts of interest

The authors declare that there is no conflict of interest.

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