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Research Article

Studies On Effect Of Supplementation Of Ascorbic Acid On Post Thaw Sperm Acrosome Integrity Of Frieswal Bull Spermatozoa

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ABSTRACT

This experiment was designed to study the effect of Ascorbic acid on post thaw sperm acrosome integrity of Frieswal bull semen during cryopreservation. For this purpose, ejaculates were collected from three Frieswal bulls using artificial vagina at biweekly interval. The semen sample which possesses more than 70 % progressive motility and above 600 million/ml spermatozoa concentration was subsequently subjected to processing for LN¬2 vapour freezing. Semen samples were extended in GEYT extender and split into 4 parts. One part was left as such (control) while the other parts contain GEYT+ Ascorbic acid at concentration of 2.5 mM (TG-I), 5.0 mM (TG-II) and 7.5 mM (TG-III). At fresh stage per cent intact acrosome. At post thaw stage semen was evaluated for per cent intact acrosome. TG-II (5.0 mM Ascorbic acid), showed significant ($p<0.05$) improvement in per cent intact acrosome in comparison to control, TG-I and TG-III. It was concluded that supplementation of Ascorbic acid improves acrosome integrity at post thaw and gives a significant effect at the concentration of 5.0 mM ascorbic acid in comparison to control, 2.5 mM and 7.5 mM concentration of ascorbic acid respectively.

INTRODUCTION

Despite the widespread use of sperm cryopreservation, it is well known that the freezing and thawing processes harm sperm due to temperature fluctuations, osmotic stress induction, and ice crystal formation (1). The cryopreservation process generally results in the loss of 40–50% of the sperm population, even with "optimized" cooling/thawing protocols (2, 3). The data obtained show that the viable subpopulation is compromised after cryopreservation as compared to fresh semen, based on a similar number of motile cells (3). It is true that sperm with poor motility have little chance of making it to the in

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vivo fertilization location and fertilizing the oocyte (4). The modification of the sperm plasma membrane resulting from cryopreservation thus inexorably impacts these normal biological processes and lowers the likelihood of successful conception. During cryopreservation, a large number of cells die or are severely damaged. In addition to these losses, a subpopulation of spermatozoa undergoes cryo capacitation process (4). The remaining non-capacitated sperm retain their ability to fertilize. According to (4), the process of "cryo-capacitation" would result in a subpopulation of sperm that has a shorter lifespan, hence decreasing the frozen sperm population sample's overall fertilization efficiency. It involves a series of events during cooling, freezing, and thawing that would cause the membrane lipids to reorganize (5). This would increase the permeability of the membrane and, as a result, predispose to premature capacitation of the sperm due to the exit of cholesterol. Another side effect observed as a consequence of the cryopreservation is the reduction of the functional and structural integrity of the sperm due to the generation of reactive oxygen species (ROS). Oxidative stress of mammalian semen during cooling can cause structural and functional damage to the sperm (6). Excessive production of oxidative oxygen metabolites could have a toxic effect through the production of free radicals, affecting sperm functionality parameters, such as motility deprivation, enzyme inactivation and plasma membrane damage (7). The detrimental effects of ROS could cause that the sperm be unable to fertilize the oocyte. The oxidative stress is caused by the formation of large amounts of ROS or molecules that contain free radicals (RL), which are present during the handling and manipulation of the ejaculate and filled straws during cooling, freezing and thawing. Ascorbic acid (Vitamin C) is a non-enzymatic water-soluble antioxidant which contributes 65% of the antioxidant capacity of seminal plasma (1). Ascorbic acid is an excellent antioxidant which is due to strong electron donor nature (1,8). Addition of ascorbic acid in cattle bull semen improved sperm motility and fertilizing ability. Similarly, numerous other studies are available which tested the impact of addition of ascorbic acid in in-vitro or in-vivo trials concerning bovine, boar, humans, rabbit, canine and stallion semen with controversial efficacy and usefulness (8,9).

MATERIALS AND METHODS

Experimental Animal

The present study was undertaken at the Semen Freezing Laboratory, Division of Cattle Physiology and Reproduction, ICAR-Central Institute for Research on Cattle, Meerut Cantt, India. The institute is located at 247 m elevation, at latitude of 29º N and a longitude of 78° E. It has a monsoon-influenced humid subtropical climate characterized by hot summers and cold winters. Three healthy Frieswal (Holstein-Friesian × Sahiwal) breeding bulls were maintained under uniform feeding and housing conditions with an average weight of 500-600 Kg were utilized for the study.

Semen collection, extension, grouping and supplementation

The ejaculates were collected early in the morning between 8 to 9 AM in graduated glass tubes attached to the artificial vagina as per regular standard practice. One false mount was given to every bull before actual collection. A total of 18 ejaculates with an initial progressive motility (IPM) of ≥70% and a concentration of over 500 × 106 sperm/ml were selected for the experiment. The extender used for semen dilution was GEYT with Tris (3.02 g), citric acid (1.67 g), fructose (1.25 g), penicillin (100,000 IU), streptomycin (100 mg), glycerol (7 ml), and egg yolk (20 ml) in Millipore water. The concentration of sperm cells was kept at approximately 80 million progressive motile sperm cells/ml in all the groups. The

parameter assessed at fresh stage was acrosome integrity. Simultaneously, each sample was divided into four equal groups, and the experiment consisted of three treatment groups (TGs) and one control group (CG). In CG, the spermatozoa were frozen in GEYT media without any supplementation. In the three treatment groups, namely TG-I, TG-II, and TG-III, the spermatozoa were frozen in GEYT media supplemented with different concentrations of ascorbic acid. The final concentration of ascorbic acid in TG-I, TG -II and TG-III was kept at 2.5 mM, 5.0 mM and 7.5 mM, respectively. The ascorbic acid was procured from Sigma Aldrich Chemicals Pvt. Ltd.

Evaluation of sperm Acrosome integrity

Acrosomal integrity was assessed using the Giemsa solution, as per the standard protocol (3 Watson, 1975). For examination of percent acrosome integrity, a thin smear of extended semen was made on a glass slide, air-dried and fixed in Hancock's fixative for 20 min. Thereafter, the slides were removed and washed under running tap water. The washed slides were dipped into Coplin staining jars filled with Giemsa stain for 4 hrs. Then, slides were rinsed under water, air-dried and examined under oil immersion (1000 x). Statistical analysis The statistical analysis of the data was done as per the standard procedures. The data recorded in percent values was subjected to angular transformation before the analysis. Analysis of variance (ANOVA) was used to compare means using SPSS 20.0 (SPSS Inc., Chicago, USA) statistical software. Statistical significance was tested at a 5% ($p < 0.05$) probability level, and the difference between means was compared using Duncan's multiple range test.

RESULTS

At the fresh stage mean percentage of intact acrosome was 77.50±1.20. At the post-thaw stage, the mean percentage of spermatozoa with intact acrosome in control and TG-I, TG-II and TG-III were 62.17±1.98, 67.17±1.73, 69.56±1.14 and 60.67±1.60, respectively. As compared to fresh stage the mean percentage of spermatozoa with intact acrosome was significantly reduced after freeze-thaw process. Supplementation of ascorbic acid significantly ($p<0.05$) improved the sperm acrosome integrity in TG-I & TG-II as compared to control & TG- III. However, addition of ascorbic acid in TG-III did not show any significant improvement as compared to the control group.

DISCUSSION

In our study, the supplementation of ascorbic acid at a concentration of 2.5 mM (TG-I), 5.0 mM (TG-II) considerably improved the acrosome integrity and ameliorated the effects of cryopreservation on sperm acrosomal membrane. The studies were comparable with the previous studies on bull semen (1, 8, 9) implying that ascorbic acid supplementation could exert its protective action on the crossbred bull spermatozoal cells. It was also observed that ascorbic acid supplementation at a concentration of 7.5 Mm (TG-III) could not bring any notable change in acrosome integrity as compared to control. This might be due to pro-oxidant nature of ascorbic acid at higher concentration. Present study was in agreement with (1, 8, 9).

CONCLUSION

The semen cryopreservation is influenced by several factors and is known to damage sperm in a variety of ways. Such damages lead to a decrease in survival rate and acrosome integrity. The supplementation of ascorbic acid at a concentration of 2.5 mM (TG-I), 5.0 mM (TG-II) considerably improved the acrosome integrity and ameliorated the effects of cryopreservation on sperm acrosomal membrane integrity.

Relevant conflicts of interest/financial disclosures

The authors declare that the research was conducted in the absence of any commercial or

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