Original Research Article

Effects of Ascorbic Acid as Antioxidant Semen Additive in Cryopreservation of Cross-bred Cattle Bull Semen

Padamveer Singh\(^1\), Sanjay Agarwal\(^1\)*, Harjyote Singh\(^1\), Satbir Singh\(^2\), Pawan Kumar Verma\(^3\), Mohd Shaheem Butt\(^1\) and Utsav Sharma\(^1\)

\(^1\)Division of Veterinary Gynaecology & Obstetrics, F.V.Sc. & A.H., S.K.U.A.S.T.-J., R. S. Pura-181102, Jammu, India
\(^2\)Frozen Semen Laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu, India
\(^3\)Division of Veterinary Pharmacology and Toxicology, F.V.Sc. & A.H., S.K.U.A.S.T.-J., R. S. Pura-181102, Jammu, India

*Corresponding author

A B S T R A C T

The present study was jointly undertaken at the Frozen Semen Laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu and Division of V.G.O., F.V.Sc & A.H., SKUAST- J, R.S.Pura, during the period between December 2017 and May 2018. This investigation was carried out with the objective to study the effect of antioxidant semen additive ascorbic acid on cryopreservation of semen. Semen samples (n=10) from mature cattle bull stationed at Frozen semen laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu, were used to evaluate the effect of ascorbic acid additive at post-dilution and at post thaw stage. The semen sample was extended with Tris-Egg-Yolk-Citric-acid-Fructose-Glycerol (TEYCAFG) extender and were split into two groups: Group 1: TEYCAFG without any additive/ control and group 2: TEYCAFG + Ascorbic acid (5mM). Progressive motility, live spermatozoa, acrosomal integrity, sperm abnormality, hypo-osmotic swelling test (HOST) was evaluated at both post-dilution and post-thaw stage. Whereas, oxidative stress tests viz. malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) were evaluated at only post-thawed stage. Group 2 i.e. ascorbic acid group, showed significant (p<0.05) increased live spermatozoa, acrosomal integrity, sperm abnormality, hypo-osmotic swelling test (HOST) was evaluated at both post-dilution and post-thaw stage. Whereas, oxidative stress tests viz. malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) were evaluated at only post-thawed stage. Group 2 i.e. ascorbic acid group, showed significant (p<0.05) increased live spermatozoa, acrosomal integrity and HOST positive spermatozoa, while significant (p<0.05) decreased sperm abnormalities in post-thawed semen. In oxidative stress evaluation the MDA level was significantly (p<0.05) decreased, whereas, SOD levels significantly (p<0.05) increased in group 2 in comparison to control group. It was concluded that addition of ascorbic acid (5 mM) as semen additive improves semen quality and minimize oxidative stress to the spermatozoa during cryopreservation of semen.

Keywords
Semen, Cattle bull, Ascorbic acid, Oxidative stress

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Introduction
The milk production in India was 187.7 Million Tonnes (MT) in year of 2018-19 (NDDB, 2020) in which contribution of Jammu and Kashmir (J&K) is 254 MT. The cattle population in the country as per 2019 census were 192.5 million out of total bovine
population (302.3 millions) in J&K total cattle including cross-bred and indigenous were 1231 (in thousands). To meet the objective of sustainable milk production, special attention is required for improving the gene pool of the animals. The best proven way to improve gene pool is artificial insemination of the animals with frozen semen from superior bulls, thus it is only possible by making available sufficient number of frozen doses from superior bulls.

Now-a-days the bulls frozen semen has been widely used in artificial insemination (AI) but the results of conception is about 50 per cent. This might be due to freezing and thawing processes that leads to the generation of reactive oxygen substances (ROS) that impair post-thaw motility, viability, intracellular enzymatic activity, fertility and sperm functions (Aitken et al., 1998; Zhao and Buhr, 1995; White, 1993).

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 (Betteridge, 2000). Oxidative Stress installed at the level of tissues, organs or organelles is derived from the imbalance between the production and elimination of reactive oxygen species. Spermatozoa and seminal plasma possess an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage. However, this anti-oxidant capacity in sperm cells, due to the small cytoplasmic component that contains antioxidants to scavenge oxidants, is limited (Lapointe and Bilodeau, 2003; Aurich et al., 1997; Storey, 1997). Addition of antioxidants to the freezing media like ascorbic acid, catalase, caffeine, glutathione, taurine etc. in semen diluent prevent the damage induced by free radicals to spermatozoa during freezing as well as improves the fertility of the semen (Agarwal et al., 2004).

Ascorbic acid or Vit C concentration in seminal plasma exceeds 10 times more than that in blood plasma (364 compared with 40 μmol/L) (Tariq et al., 2015). It is an antioxidant substance, which is normally present in the epididymal fluid and seminal plasma of several species (Chinoy, 1972) which protects sperm from Reactive Oxygen Species (Buettner, 1993). Vit.C or ascorbic acid specifically reduces oxygen radicals, neutralizes ROS and regenerates other antioxidants system and helps in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA (Fraga et al., 1991).

Though few research using ascorbic acid as an semen additive has been previously reported but differences exists among ascorbic acid concentrations used, species, breed, dilutor composition, freezing method etc. but such research in the cross-bred bull semen of Jammu and Kashmir region is completely missing.

Thus keeping in view the aforesaid facts the present study was designed with the objective to evaluate the oxidative stress to the spermatozoa during cryopreservation of semen and the effects of ascorbic acid as semen additive.

**Materials and Methods**

The present study was jointly undertaken at the Frozen semen laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu and Division of V.G.O., F.V.Sc & A.H., SKUAST- J, R.S.Pura, during the period between
December 2017 and May 2018. Semen samples (n=10) were collected randomly from mature cattle bull stationed at Frozen semen laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu, India (32.73º N, 74.87º E, altitude 300 m). Semen was collected twice a week from each bull by artificial vagina method. The semen ejaculates were initially evaluated for volume, mass activity, spermatozoa concentration and progressive motility. The ejaculates qualifying the minimum initial standards were further processed. After initial assessment of semen, the selected samples were extended with pre-warmed (37°C) Tris egg yolk citric acid fructose glycerol (TEYCAFG) extender so that each ml of extended semen contained at least 40 x 10^6 spermatozoa (Anel et al., 2003).

After extension, the semen samples were divided into two parts. The first part was considered as group 1: TEYCAFG without any additive/ control and group 2: TEYCAFG + Ascorbic acid (5mM). The pH was adjusted within the range 7.2 to 7.4. The semen samples were filled in 0.5 ml capacity, polyvinyl straw (IMV, France). Equilibration was done in the cold handling unit maintained at 4º C for 4 hours. Freezing of the straws was done by programmable biofreezer (Digit Cool-530, IMV Technologies, France) with rate freezing from 4°C to -10°C @ 5°C, -10°C to -100°C @ 40°C, -100°C to -140°C @ 20°C, after attainment of -140°C temperature the straws were removed from the racks shifted to the pre-cooled goblets and plunged into liquid nitrogen and finally stored in liquid nitrogen tank where they were stored for definite period of time for future evaluation.

Total ejaculates (n=10) were evaluated at two stages of the semen processing viz. post-dilution and post-thaw stage, for per cent progressive motility, live spermatozoa, acrosomal integrity, sperm abnormalities and hypo-osmotic swelling test. Whereas, only at post-thaw stage for oxidative stress test viz. MDA, SOD and Catalase. Thawing of frozen semen was done at 37º C for 30 seconds.

Volume was measured by graduated collecting tube, mass activity was graded as described by Tomar et al., (1966). The concentration of the spermatozoa (millions/ml) was determined by Accucell photometer (IMV Technologies, France). Progressive motility, live spermatozoa and acrosome integrity were assessed as per Salisbury et al., (1978); HOST test was performed as described by Jeyendran et al., (1984); sperm abnormalities were assessed by Eosin-nigrosin stain with slight modification as described by Kumar (1993). Oxidative stress test viz. Malondialdehyde (MDA) was determined as described by Shafiq-ur-Rehman (1984); Catalase as per Aebi (1983) and Superoxide dismutase (SOD) as described by Marklund and Marklund (1974). The results were analysed statistically using Analysis of Variance (ANOVA) (Snedecor and Cochran, 1989).

### Results and Discussion

#### Progressive motility

The per cent progressive motility (Mean ± SE) post-dilution and post-thawing in control group were 67.0 ± 1.52 and 48.0 ± 2.49; whereas in ascorbic acid group were 68.0 ± 1.33 and 50.0 ± 2.11, respectively (Table 1.0). Perusal of the table (1.0) it was observed that the progressive motility (%) differed none significantly between post-diluted control and ascorbic acid group as well as post-thaw control and ascorbic acid group.

In the present study in control group the per cent progressive motility of diluted semen (Table 1.0) was 67.0 ±1.52 which was in agreement with the finding of Pathak et al., (1990) who reported per cent progressive motility as 65.30 ± 1.20 per cent in diluted samples.
semen of cattle bull. Whereas, in the control group per cent post thaw motility (Table 1.0) was in intermediate range in comparison to previous reports, which was less than reported by Kishore, 2009 (80.74); Pawshe et al., 2016 (61.7 ± 2.6); Uysal et al., 2007 (55.5 ± 2.5) and Ulfina and Raina, 2003 (55.34 ± 1.02), while it was higher than reported by Hu et al., 2010 (36.88 ± 1.53) and Li et al., 2016 (14.7 ± 1.4).

The main reason for the decrease in the per cent post thaw progressive motility might be due to cryopreservation damage, ROS production and damage caused due to formation of ice crystal formation in mitochondria and Axosomes during cryopreservation that impairs sperm motility.

In the present study in ascorbic acid group the values of per cent progressive motility of diluted semen (Table 1.0) was 68.0 ± 1.33, which was in agreement with the finding of Mittal et al., 2014 who reported as 66.59 ± 0.99, while it was lesser than as reported by Rao et al., 2017 (78.05 ± 0.02) and Sandeep et al., 2015 (73.33 ± 1.07). Whereas, per cent progressive motility of post thaw semen (Table 1.0) was 50.0 ± 2.11, which was higher than as reported by Rao et al., 2017 (46.83 ± 0.01) and Sandeep et al., 2015 (45.62 ± 0.69) and lower than the finding of Sohail et al., 2015 (62.73 ± 2.80) and Mittal et al., 2014 (56.75 ± 0.75).

**Live spermatozoa**

The per cent live spermatozoa (Mean ± SE) post-dilution and post-thawing in control group were 74.4 ± 1.82 and 50.8 ± 1.17; whereas in ascorbic acid group were 72.4 ± 1.69 and 55.4 ± 0.88, respectively (Table 1.0). Perusal of the table (1.0) it was observed that the live spermatozoa (%) differed non significantly (p<0.05) difference in live spermatozoa was observed between control and ascorbic acid group.

In the present study in the control group values of per cent live spermatozoa in diluted semen (Table 1.0) was 74.4 ± 1.82, which was in agreement with the findings of Keshava (1996) who reported 74.28 and Abdul-khalek et al., 2008 as 73.0 ± 1.3. Whereas per cent live spermatozoa in post thaw semen (Table 1.0) was 50.8 ± 1.17 which was less than reported by Bhalde et al., 1991 (57.64 ± 0.78), Rao et al., 2017 (56.24 ± 0.01) and Abdul-khalek et al., 2008 (66-73 per cent). Decreasing proportion of live spermatozoa in post thaw semen may be due to cryo-damage caused to formation of ice crystals in extra and intracellular environment, increasing solute concentration (Mazur, 1984) and sperm susceptibility for freezing and thawing temperature, ROS production and lipid peroxidation (Bucak et al., 2008).

In the present study in ascorbic acid group the value of per cent live spermatozoa of diluted semen was 72.4 ± 1.69 which was in agreement with the findings of Sandeep et al., 2015 (76.21 ± 1.01). Whereas per cent live spermatozoa in post thaw semen (Table 1.0) was 55.4 ± 0.88 which was higher than the findings of Rao et al., 2017 (51.92 ± 0.02) and Sandeep et al., 2015 (48.21 ± 0.75), while lesser than the finding of Sohail et al., 2015 (60.43 ± 3.17).

**Acrosomal integrity**

The per cent acrosomal integrity (Mean ± SE) post-dilution and post-thawing in control group were 74.4 ± 1.82 and 50.8 ± 1.17; whereas in ascorbic acid group were 72.4 ± 1.69 and 55.4 ± 0.88, respectively (Table 1.0). Perusal of the table (1.0) it was observed that the acrosomal integrity (%) differed non significantly between post-diluted control and ascorbic acid group, whereas, in post-thaw a
significantly between post-diluted control and ascorbic acid group, whereas, in post-thaw a significant (p<0.05) difference in acrosomal integrity was observed between control and ascorbic acid group.

In the present study in control group the values of per cent acrosomal integrity of diluted semen (Table 1.0) was 79.7 ± 1.52, which was in agreement with the finding of Lone et al., 2017 (80.17 ± 3.26), it was less than the finding of Rao et al., 2017 (85.77 ± 0.01). Whereas, per cent acrosomal integrity of post thaw semen was 53.9 ± 0.94 which was in agreement with the finding of Rao et al., 2017 (56.24 ± 0.01), however it was higher than the finding of Sandeep et al., 2015 (36.25 ± 0.91) and Paudel et al., 2010 (31.5 ± 1.3). The decrease in acrosomal integrity at various stages may be due to loss of component of plasma membrane and loss of plasmalemma over entire acrosome during freeze-thawing.

In the present study in ascorbic acid group the values of per cent acrosomal integrity of diluted semen (Table 1.0) was 78.7 ± 1.78 which is lower than the finding of Rao et al., 2017 (85.77 ± 0.01) and Mittal et al., 2014 (81.75 ± 0.47), while higher than the finding of Sandeep et al., 2015 (76.21 ± 1.01). Whereas, per cent acrosomal integrity of post thaw semen was 64.3 ± 2.42 which was in agreement with the finding of Rao et al., (2017) who reported as 63.57 ± 0.01, while higher value was observed by Mittal et al., 2014 (71.59 ± 0.48) in their study and lower finding were observed by Sandeep et al., 2015 (48.21 ± 0.75) and Paudel et al., 2010 (35.9 ± 1.4).

**Sperm abnormalities**

The per cent sperm abnormalities (Mean ± SE) post-dilution and post-thawing in control group were 7.2 ± 0.63 and 16.8 ± 0.61; whereas in ascorbic acid group were 6.8 ± 0.83 and 13.2 ± 0.79, respectively (Table 1.0). Perusal of the table (1.0) it was observed that the sperm abnormalities (%) differed non significantly between post-diluted control and ascorbic acid group, whereas, in post-thaw a significant (p<0.05) difference in sperm abnormalities was observed between control and ascorbic acid group.

In the present study in control group the values of per cent sperm abnormalities in diluted semen (Table 1.0) was 7.2 ± 0.63, which was in agreement with the finding of Rao et al., (2017) who reported as 7.28 ± 0.02, however it was higher than as reported by Mittal et al., 2014 (4.91 ± 0.14), while it was lower than as reported by Gupta et al., 1990 (9.14 ± 0.09). Whereas, per cent post thaw sperm abnormalities was 16.8 ± 0.61 which was in agreement with the finding of Rao et al., (1999) who reported a range of 15.66 ± 0.39 to 16.75 ± 0.37, while it was higher than as reported by Mittal et al., 2014 (7.75 ± 0.17), Sariözkan et al., 2009a (14.93 ± 1.07) and Sariözkan et al., 2009b (15.0 ± 1.1), whereas lower than the finding of Gupta et al., 1990 (17.63 ± 1.99) and Rao et al., 2017 (18.36 ± 0.04).

In the present study in ascorbic acid group the value of per cent sperm abnormalities of diluted semen (Table 1.0) was 6.8 ± 0.83 which was in agreement with the finding of Rao et al., (2017) who reported as 6.78 ± 0.03, while lower value of per cent sperm abnormalities was observed in the finding of Mittal et al., 2014 (6.84 ± 0.12). Whereas, the values of post thaw semen was 13.2 ± 0.79 which was lower than the finding of Rao et al., 2017 (17.62 ± 0.01), while higher than the finding of Mittal et al., 2014 (6.84 ± 0.12).

**Hypo-osmotic swelling test (HOST)**

The hypo-osmotic swelling test positive spermatozoa per cent (Mean ± SE) post-
dilution and post-thawing in control group were 71.0 ± 1.10 and 45.4 ± 0.93; whereas in ascorbic acid group were 71.5 ± 1.69 and 49.4 ± 0.87, respectively (Table 1.0). Perusal of the table (1.0) it was observed that the hypo-osmotic swelling test positive spermatozoa (%) differed non significantly between post-diluted control and ascorbic acid group, whereas, in post-thaw a significant (p<0.05) difference in hypo-osmotic swelling test positive spermatozoa was observed between control and ascorbic acid group.

In the present study in control group the per cent hypo-osmotic swelling test (HOST) positive spermatozoa of diluted semen (Table 1.0) was 71.0 ± 1.10 which was in agreement with the findings of Rao et al., (2017) who reported as 72.54 ± 0.01 and Lone et al., (2017) as 70.91 ± 5.92, however lower values was observed by Kumar et al., 2018 (62.11 ± 0.89). Whereas, in post thaw semen the values of hypo-osmotic swelling test (HOST) positive spermatozoa (Table 1.0) was 45.4 ± 0.93, which was agreement with the finding of Kumar et al., (2018) who reported as 45.94 ± 1.33 and Sariözkın et al., (2009a) as 43.70 ± 1.96, while lower values were observed by Paudel et al., 2010 (39.6 ± 1.3), Rao et al., 2017 (38.73 ± 0.01) and Taraphdar, 1999 (36.52) and higher values was observed by Lone et al., 2017 (51.30 ± 4.43) and Sohail et al., 2015 (49.97 ± 3.62). The structural changes produced in the post thaw sperm cells membrane are primarily linked to altered abilities for energy sourcing which later on influence both cellular metabolism and other sperm functions (Dziekonska et al., 2009; Gillan et al., 2004).

In the present study in ascorbic acid group the values of per cent HOST positive of diluted semen has been reported as 74.36 ± 0.01 in crossbred bull with 5mM of ascorbic acid (Rao et al., 2017) which was slightly higher than our present study value of per cent HOST positive i.e. 71.5 ± 1.69 (Table 1.0). In the present study the value of per cent HOST positive of post thaw semen of ascorbic acid group (Table 1.0) was 49.4 ± 0.87 which was slightly lower than the finding of Sohail et al., 2015 (51.37 ± 3.98), while higher than the finding of Rao et al., 2017 (42.27 ± 0.02) and Paudel et al., 2010 (41.0 ± 1.3).

**Table.1.0** The effect of ascorbic acid on different physio-morphological characteristics at post-dilution and post-thaw semen of cross-bred bulls

| Parameter (%) | Stage      | Control             | Ascorbic acid (5mM) |
|---------------|------------|---------------------|---------------------|
| **Progressive motility** | Post-dilution | 67.0 ± 1.52<sup>a</sup> | 68.0 ± 1.33<sup>a</sup> |
|               | Post-thaw  | 48.0 ± 2.49<sup>a</sup> | 50.0 ± 2.11<sup>a</sup> |
| **Live spermatozoa** | Post-dilution | 74.4 ± 1.82<sup>a</sup> | 72.4 ± 1.69<sup>a</sup> |
|               | Post-thaw  | 50.8 ± 1.17<sup>a</sup> | 55.4 ± 0.88<sup>b</sup> |
| **Acrosomal integrity** | Post-dilution | 79.7 ± 1.52<sup>a</sup> | 78.7 ± 1.78<sup>a</sup> |
|               | Post-thaw  | 53.9 ± 0.94<sup>a</sup> | 64.3 ± 2.42<sup>b</sup> |
| **Sperm abnormalities** | Post-dilution | 7.2 ± 0.63<sup>a</sup> | 6.8 ± 0.83<sup>a</sup> |
|               | Post-thaw  | 16.8 ± 0.61<sup>a</sup> | 13.2 ± 0.79<sup>b</sup> |
| **HOST** | Post-dilution | 71.0 ± 1.10<sup>a</sup> | 71.5 ± 1.69<sup>a</sup> |
|               | Post-thaw  | 45.4 ± 0.93<sup>a</sup> | 49.4 ± 0.87<sup>b</sup> |

Values are given as mean ± SE of 10 animals unless and otherwise stated
Values bearing different superscripts in a row differ significantly (p<0.05)
Table 2.0 The effect of ascorbic acid on malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) activity in post-thawed semen of cross-bred bulls

| Oxidative stress test | Control        | Ascorbic acid (5mM) |
|-----------------------|----------------|---------------------|
| MDA (nmol/10^8 Spermatozoa) | 3.49 ± 0.19^a | 1.70 ± 0.04^b |
| CAT (µmol/10^8 Spermatozoa) | 1.18 ± 0.36^a | 1.31 ± 0.73^a |
| SOD (U/10^8 Spermatozoa) | 0.95 ± 0.06^a | 1.31 ± 0.05^b |

Values are given as mean ± SE of 10 animals unless and otherwise stated
Values bearing different superscripts in a row differ significantly (p<0.05)

Malondialdehyde (MDA)

The MDA levels (Mean ± SE) post-thaw semen samples in control group and ascorbic acid group were 3.49 ± 0.19 and 1.70 ± 0.04, respectively (Table 2.0). Perusal of the table (2.0) it was observed that MDA levels in post-thaw semen samples differed significantly (p<0.05) between control and ascorbic acid group.

The MDA level (nmol H_2O_2 produced/ 10^8 spermatozoa) of post thaw semen in 712.1 ± 49.1 nmol MDA level/10^9 spermatozoa in crossbred bull (Paudel et al., 2010), 496.02 ± 39.28 nmol MDA level/10^9 spermatozoa in buffaloo bull (Lone et al., 2017). In the present study MDA level (nmol H_2O_2 produced/ 10^8 spermatozoa) in post thaw semen of control group (Table 2.0) was 3.49 ± 0.19 which was lower than the value observed by Paudel et al., 2010 and Lone et al., 2017. The finding of other scientists could not be compared with our finding due to disparity in method of evaluation and units of measurement.

In the present study, the MDA level (nmol H_2O_2 produced/ 10^8 spermatozoa) post thaw semen of ascorbic acid added group (Table 2.0) was 1.70 ± 0.04. In murrah buffalo bull with 2.5mM of ascorbic acid (Sandeep et al., 2015) observed the value of MDA level (concentration ng/ 120 million spermatozoa) in post thaw semen was 521.16 ± 8.23, which was higher than the present study finding, however, Paudel et al., 2010 observed the level of MDA production (nmol/10^9 spermatozoa) in crossbred bull with 10mM concentration of ascorbic acid in post thaw semen which is higher than the value of our present investigation.

Catalase

The catalase levels (Mean ± SE) post-thaw semen samples in control group and ascorbic acid group were 1.18 ± 0.36 and 1.31 ± 0.73, respectively (Table 2.0). Perusal of the table (2.0) it was observed that catalase levels in post-thaw semen samples differed non significantly between control and ascorbic acid group.

The catalase activity (µmol H_2O_2 decomosed/ min/ 10^8 spermatozoa) in post thaw semen has been reported as 1.16 ± 0.82 (U/ml) in Qinchuan bulls (Zhao et al., 2015), 23.36 ± 0.25 in rainy season, 24.25 ± 0.30 winter season and 24.22 ± 0.56 in summer season, respectively in buffalo bull (Sharma et al., 2016). In the present study catalase activity (µmol H_2O_2 decomosed/ min/ 10^8 spermatozoa) in post thaw semen of control group (Table 2.0) was 1.18 ± 0.31 which was higher than finding of Zhao et al., (2015).
whereas lower than the finding of Sharma et al., (2016). There was reduction in the catalase activity with the level of freezing. CAT activity reduced at post thaw stage when compared to pre freeze stage (Lone et al., 2017; Kadirvel et al., 2009). CAT is an enzymatic antioxidant found in all living organisms exposed to oxygen, which decomposes harmful peroxides and converts them into water and oxygen (Chelikani et al., 2004).

In the present study, the level of Catalase (µmol H$_2$O$_2$ decomosed/ min/ 10$^8$ spermatozoa) in post thaw semen (Table 2.0) was 1.31 ± 0.73. The CAT activity reduced at post thaw stage when compared to pre freeze stage (Lone et al., 2017) and the reduction in the catalase activity also reported by Kadirvel et al., (2009).

**Superoxide dismutase (SOD)**

The SOD levels (Mean ± SE) post-thaw semen samples in control group and ascorbic acid group were 0.95 ± 0.06 and 1.31 ± 0.05, respectively (Table 2.0). Perusal of the table (2.0) it was observed that SOD levels in post-thaw semen samples differed significantly (p<0.05) between control and ascorbic acid group.

The level of Superoxide dismutase (Unit/ 10$^8$ spermatozoa) in post thaw semen have been reported as 104.02 ± 26.34 (µkat/g protein) in cattle bull (Sariözkan et al., 2009a), 7.2 ± 1.8 (U/g protein) in cattle bull semen (Sariözkan et al., 2009b), 3.50 ± 0.19 in cattle and 1.98 ± 0.09 in buffalo bulls (Nair et al., 2006) and 0.16 ± 0.03 (units/mg protein) in buffalo bulls (Lone et al., 2017). The level of Superoxide dismutase (Unit/ 10$^8$ spermatozoa) in present study in post thaw semen of control group (Table 2.0) was 0.95 ± 0.06 which was lower than the finding of Nair et al., (2006), however it was higher than the finding of Lone et al., (2017). The mean SOD activity was reduced at post-thaw stages, when compared to pre-freeze. This decline in activity of SOD may be due to utilisation of SOD in neutralizing superoxides during freeze-thaw process SOD activity was reduced to 50% in Holstein Friesian bulls during cryopreservation (Bilodeau et al., 2000). Superoxide is a free radical which is converted to oxygen and hydrogen peroxide by the dismutation action of antioxidant enzyme superoxide dismutase (Tariq et al., 2015).

In the present study, the level of Superoxide dismutase (Unit/ 10$^8$ spermatozoa) in post thaw semen of ascorbic acid added group (Table 2.0) was 1.31 ± 0.05. The mean SOD activity was reduced at post-thaw stages, when compared to pre-freeze. This decline in activity of SOD may be due to utilisation of SOD in neutralizing superoxides during freeze-thaw process SOD activity was reduced to 50% in Holstein Friesian bulls during cryopreservation (Bilodeau et al., 2000). Superoxide is a free radical which is converted to oxygen and hydrogen peroxide by the dismutation action of antioxidant enzyme superoxide dismutase (Tariq et al., 2015).

In conclusion, the present study with ascorbic acid (5mM) as the semen additive may be used for the cryopreservation of the semen. The parameters of semen quality improved in the ascorbic acid added cryopreserved semen in comparison of control group. The oxidative stress to the semen is also minimized after addition of the ascorbic acid. The in vitro fertility assessed by hypo-osmotic swelling test also indicates for the better quality and fertility in ascorbic acid group. However, the improved semen quality actually results in improved fertility rate in the cattle population should be validated.
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