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Effect of different Anti-oxidants on the Acrosome integrity and Sperm membrane Integrity (HOST) of Deccani ram sperm preserved at 5 ^{o}c

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Abstract
A study was conducted to assess the influence of Ascorbic acid, Butylated hydroxy toluene (BHT) and Vit E on semen quality parameters of Deccani rams. Semen was collected from eight Deccani rams twice a week and one twenty eight collections (sixteen collections per ram) were utilised in this study. After collection of semen sample, each semen sample diluted 1:3 with Tris citrate fructose egg yolk (TCFEY) extender then split into four equal parts first one added with Ascorbic acid (2mg/ml), second part added with Butylated hydroxy toluene (BHT) at the rate of 2mM/ml, third part added with Vitamin E at the rate of 2mg/ml and the fourth part was considered as control without any addition. And acrosome integrity and HOST (Hypo osmotic swelling test) positive sperms were evaluated at an interval of 24, 48 and 72hrs at refrigerated temperature (5 °c). The results were significantly ($P< 0.05$) better in extender supplemented with BHT, Vit E group than the Ascorbic acid and control group. But Ascorbic acid supplemented group better when compared with those of control diluent at different hours of incubation. The results revealed that supplementation of Ascorbic acid, BHT and Vit E to Tris citrate fructose egg yolk (TCFEY) extender improve semen quality in Deccani rams.

Keywords: Ascorbic acid, butylated hydroxy toluene, Vit E, refrigerated temperature and Deccani ram

Introduction
Preservation of livestock semen requires a reduction or arrest of the metabolism of spermatozoa, thereby prolonging their fertile life. This is commonly achieved by cryopreservation, but semen may also be stored in a liquid (unfrozen) state, using reduced temperatures or other means to depress metabolism (reviewed by Maxwell and Salamon 1993) [6]. However, metabolism is not completely arrested during liquid storage at reduced temperatures; the main changes which occur include an irreversible reduction in motility, morphological integrity and fertility of spermatozoa. These changes may be contributed to by the accumulation of the toxic products of metabolism and, more importantly, of reactive oxygen species (ROS) formed through the univalent reduction of oxygen (e.g. superoxide anion, hydroxyl radical and hydrogen peroxide) (Misra and Fridovich 1972) [9].
Ejaculated ram semen contains appreciable amounts of superoxide dismutase and much lower concentrations of glutathione peroxidase and catalase (Abu-Erreish *et al*. 1978; Mann and Lutwak-Mann 1981) [8, 9], but their concentration may be considerably reduced by dilution of semen. There seem to be no studies on the effects of antioxidants added to ram semen to protect the spermatozoa from ROS during liquid storage. In the present study, the antioxidants Ascorbic acid, BHT and Vit E were included in diluents and their effect on acrosome integrity and HOST positive sperms were examined after liquid storage of semen.

Materials and Method
Subsequent to dilution of semen in Tris-citric acid-fructose-egg yolk (TCFEY) dilutor, each semen sample was divided into four groups.

Groups were as follows
Group 1: Tris citrate fructose egg yolk diluent with 2mg of ascorbic acid
Group 2: Tris citrate fructose egg yolk diluent with 2mM of Butylated hydroxy toulene
Group 3: Tris citrate fructose egg yolk diluent with 2mg of Vit E
Control: Tris citrate fructose egg yolk diluent without antioxidant.

Acrosomal Integrity

The intact acrosome of spermatozoa was essential until they bind to Zona pellicuda. The spermatozoa may be highly motile but not fertile due to acrosomal damage. The acrosomal damage (percent damaged acrosomes in fresh, diluted and chilled semen) was studied in Giemsa stained smears according to the method described by Barth and Oko (1989) [2].

Procedure

Semen sample was diluted in 2.9% sodium citrate buffer (freshly prepared) to 1:4 in a test tube. A smear was drawn on clean grease free slide and air dried. The slides were put into 5% formaldehyde solution for fixing at 37 ^{0}C for 30 min (Campbell *et al.*, 1960) [4]. The slides were removed from the solution, washed in running tap water and air dried for further processing. The working solution of Giemsa was prepared mixing Giemsa's stock-3 ml, SPS-2 ml and dist. water- 45 ml in a cup linger and warmed at 37 ºC for 30 min. The smeared slides of spermatozoa were put into the working solution and kept at 37 ºC for 3 hrs. The slides were removed from the stain and washed in running tap water and finally air dried. The counting of intact, partially damaged and fully damaged acrosome was carried out in oil immersion microscope at 1000X (10X100) magnification.

Host

HOS test was developed to evaluate the functional integrity of sperm membrane. The HOS test has become a routine evaluation employed to assess the integrity of the plasma membrane and possesses high relevance in assisted reproductive technologies (World Health Organization, 2010) [8].

Procedure

One ml of pre- incubated hypo-osmotic solution (150 mOsm/L) was mixed with 0.1 ml of extended semen in a small test tube. A control was set by mixing 1 ml of control solution (300 mOsm/L) with 0.1 ml of same semen in another test tube. Both test tubes were then incubated in water bath at 37°C for 30 minutes. A drop from each solution of incubated semen was examined under phase contrast microscope at 400X (10x40) magnification for swelling (ballooning or curling) of sperm tails. A minimum of 100 spermatozoa were counted. The proportion of swollen spermatozoa in the control sample was subtracted from the proportion of swollen spermatozoa in hypo-osmotic solution. The resultant figure was considered as percentage of HOS test reactive spermatozoa.

Statistical Analysis

The data obtained in the study were analyzed statistically by using one-way analysis of Variance test (ANOVA) with the help of statistical software SPSS version 16. The post hoc analysis was performed using Duncan's multiple range tests. The level of significance was set at $P< 0.05$. The data are presented in the tables as mean ± SEM.

Results

Acrosome integrity

The average percentage of intact acrosomes of semen diluted in control, Group I, II and III chilled (5 ºC) at 24 hrs were 61.066±1.41, 70.316±0.67, 78.133±0.80 and 77.500±1.31, respectively at 48hrs were 50.28±1.03, 59.75±0.66, 67.26±0.85 and 65.93±0.84, respectively. And at 72hrs were 38.45±0.91, 48.26±0.61, 56.95±0.72 and 55.28±0.81, respectively. There is significant ($P\leq 0.05$) difference between control and group I which differ significantly with that of group II and III. While there was no significant difference ($P\geq 0.05$) between group II and III pertaining to acrosome integrity (5 ºC) at 24 h. (Table No.1) (Fig No.1)

Table 1: Show the particulars and ascorbic acid

Particulars	Control	Ascorbic acid (Group 1)	BHT (Group 2)	Vitamin E (Group 3)
At 24hrs	61.066±1.41^a	70.316±0.67^b	78.133±0.80^c	77.500±1.31^c
At 48hrs	50.28±1.03^a	59.75±0.66^b	67.26±0.85^c	65.93±0.84^c
At 72hrs	38.450±0.91^a	48.266±0.61^b	56.950±0.72^c	55.283±0.81^c

Host

The percentage of the mean sperm positive to HOS-test of semen diluted in control, Group I, II and III chilled (5 ºC) at 24 hrs were 61.216 ± 1.47, 67.800 ± 1.04, 75.100 ± 1.13 and 78.150 ± 0.88 respectively at 48hrs were 50.950±1.046, 57.733±0.54, 66.150±1.09 and 67.716±0.85, respectively. There is significant ($P\leq 0.05$) difference between control and group I which differ significantly with that of group II and III. While there was no significant difference ($P\geq 0.05$) between group II and III pertaining to HOST (5 ºC) at 24 hrs and 48hrs. (Table No. 2).

The mean percentage of the sperm positive to HOS test of semen in control, Group I, II and III chilled (5ºC) at 72 h were 40.26±1.16, 48.28±0.53, 55.65±0.355 and 58.916±1.04, respectively. There was significant ($P\leq 0.05$) difference between groups. (Table No.2) (Fig No.2)

Table 2: Difference between groups 2 and 3 pertaining to HOST (5ºC) at 24 hrs and 48hrs

Particulars	Control	Ascorbic acid (Group 1)	BHT (Group 2)	Vitamin E (Group 3)
At 24 hrs	61.216±1.47^a	67.800±1.04^b	75.100±1.13^c	78.150±0.88^c
At 48hrs	50.950±1.046^a	57.733±0.54^b	66.150±1.09^c	67.716±0.85^c
At 72hrs	40.266±1.16^a	48.283±0.53^b	55.650±0.355^c	58.916±1.04^d

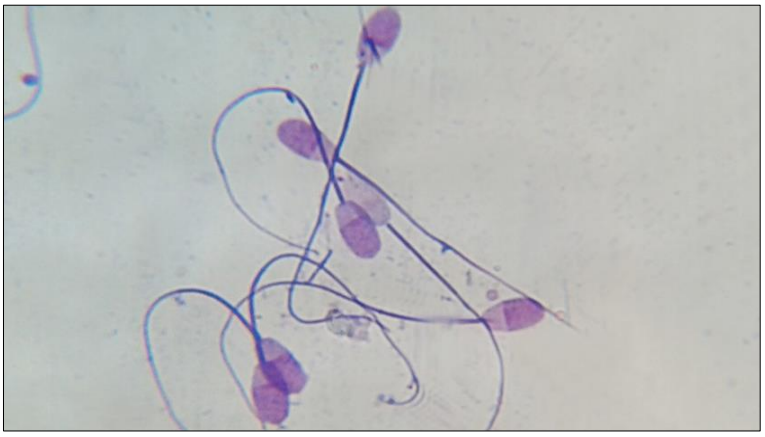

Fig 1: Photomicrograph showing Acrosomal integrity

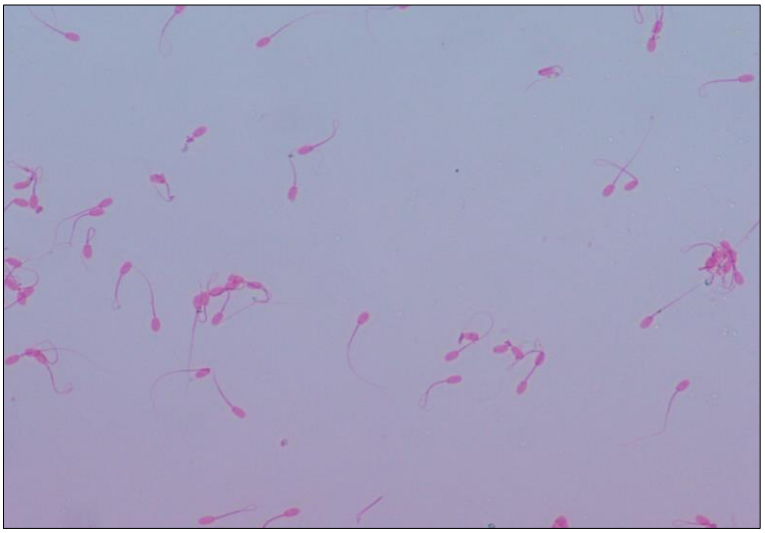

Fig 2: Host reactive sperm with tail coiling pattern

Discussion

Acrosome integrity

These results are in accordance with the studies of Bhakat *et al.,* (2011) [3] and Azawi and Hussein, (2013) [1] where addition of vitamin E resulted in a significant decrease in sperm acrosomal defects than the control group of Awassi ram semen after 120 hrs of incubation at 5˚C.

The observation on acrosome integrity were in accordance with the studies of Rather *et al.,* (2016) [7] and Bhakat *et al.,* (2011) [3] where the intact acrosome was significantly higher in the BHT group than the control group.

Similarly in ascorbic acid the results are in accordance with the findings of Azawi and Hussein, (2013) [1] where addition of vitamin C resulted in a significant decrease in sperm acrosomal defects of Awassi ram semen after 120 hr of incubation at 5˚C. Adding vitamin C to Tris diluent showed more protective effect than vitamin E.

Host

The observation on sperm membrane integrity were in accordance with the studies of Bhakat *et al.,* (2011) [3] in the Karan Fries bull semen, for all periods of incubation except after 72 hrs, Vit E were significantly better than the control. Similarly Kheradmand *et al.,* (2006) [5], adding vitamin E to the extender of ram semen during dilution is superior in maintenance of sperm membrane integrity up to 48hrs during storage at 5°C.

The observation on sperm membrane integrity were in accordance with the studies of Bhakat *et al.,* (2011) [3] in the Karan Fries bull semen, for all periods of incubation except after 72 hrs, BHT were significantly better than the control. Similarly, Rather *et al.,* (2016) [7] the percentage of HOST reacted spermatozoa was significantly higher in BHT incorporated group compared to control or ascorbic acid group.

On contradict to the present study, Rather *et al.,* (2016) [7] noticed that percentage of HOST reacted spermatozoa was significantly lower in ascorbic acid group compared to other groups. Similarly, Kheradmand *et al.,* (2006) [5] reported that ascorbic acid (0.9mg/ml) had low or no significant effect on sperm membrane integrity.

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