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Anil Manga
M.V.Sc Scholar, Department Veterinary Gynaecology and Obstetrics, PVNRTVU, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

K Venkataramana
Professor, Department Veterinary Gynaecology and Obstetrics, PVNRTVU, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

K Muralimohan
Professor, Department Veterinary Gynaecology and Obstetrics, PVNRTVU, Principal, Animal Husbandry Polytechnic College, Siddipet, Telangana, India

Corresponding Author
Anil Manga
M.V.Sc Scholar, Department Veterinary Gynaecology and Obstetrics, PVNRTVU, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Evaluation of different anti oxidants on the semen parameters of Deccani ram sperm preserved at 5 °C

Anil Manga, K Venkataramana and K Muralimohan

Abstract
The present study was conducted to determine the effects of the addition of antioxidants Ascorbic acid, BHT and Vit E in Tris-citric acid-fructose-egg yolk (TCFEY) to liquid Deccani Ram semen at refrigerated temperature (5 °C). Ejaculates were collected from eight Deccani rams by artificial vagina twice a week during non breeding season, after collection each ejaculate was diluted by Tris-citric acid-fructose-egg yolk (TCFEY), diluted semen samples was divided into four parts. The first part was added with Ascorbic acid (2mg/ml), second part added with Butylated hydroxy toluene (BHT) at the rate of 2mM/ml, Vitamin E was added at the rate of 2mg/ml to the third part and the fourth part was considered as control without any addition. The diluted semen samples were cooled gradually and preserved at 5 °C for three days. The sperms in cooled diluted semen samples were examined for motility, viability and abnormalities at 24, 48 and 72hrs of storage. the mean sperm motility percentage varied significantly ($P<0.05$) after 24hrs of storage at 5 °C which was found to be highest in Tris extender containing vitamin E followed by BHT ,Vitamin C and control group. After 48 and 72hrs of storage the individual motility was significantly highest in BHT followed by Vit E, Ascorbic acid and control. With regard to the total mean sperm viability was varied significantly highest in Tris extender containing BHT followed by Vit E, Ascorbic acid and control at 24,48 and 72hrs of storage .With regard to the mean sperm abnormalities varied significantly which was found to be lowest in Tris extender containing BHT followed by Vit E, Ascorbic acid and control at 24,48 and 72hrs of storage.

Keywords: ascorbic acid, butylated hydroxy toluene, vit E, refrigerated temperature and Deccani ram

Introduction
The survival of ejaculated sperm in seminal plasma alone is limited to a few hours. To maintain sperm for longer periods and to cool or cryo preserve semen, dilution with a protective solution is necessary (Ax *et al*., 2000) [3].

Artificial insemination with chilled-stored semen has become a technique in sheep breeding (Arthur *et al*., 1996 and Ax *et al*., 2000) [1, 3]. Efforts to improve the preservation of cooled ram semen have focused on alteration of extenders (Marti *et al*., 2003) [13] as well as the addition of specific components to maintain membrane integrity, prevent oxidative stress or preserve motility of spermatozoa in ram (Watson and Anderson, 1983; Maxwell and Stojanov, 1996 and Upreti *et al*., 1998) [20, 14, 19]. The outcome of such studies had varied success in terms of improvement of either maintenance of motility of cooled spermatozoa or fertility of cooled semen.

An important cellular system of protection against oxidative damage is DL-α tocopherol (Vitamin E), a lipophilic component that not only scavenges oxygen radicals not from within the membrane but also intercepts lipid peroxyl radicals which appear to be important in the propagation of the chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1999) [10].

Vitamin C (ascorbic acid or ascorbate) represents the major water soluble antioxidant in plasma. Vitamin C is required *in vivo* as an antioxidant in the presence of transition metal ions high concentrations of Vitamin C can act as a proxidant by donating an electron that reduces such ions to form that, in turn, can react with oxygen molecules to form oxygen radicals (Halliwell and Gutteridge, 1999; Combs, 1998; Padayatty *et al*., 2002 and Carr and frei, 2002) [10, 8, 15, 9].

There is evidence that Butylated hydroxy toluene (BHT), as a Phenolic antioxidant, acted as a membrane lipid perturbant which prevented or substantially reduced the permeability changes of sperm plasma membrane when the cell was cold-shocked (Hammerstedt *et al*., 1976) [11].

Materials and Methods
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

Individual motility

The individual motility of sperm was observed under microscope (Verberckmoes *et al.,* 2001) [21] and assessed by placing a cover slip on a drop of diluted semen (10µl semen mixed with 200µl normal saline was used for fresh semen evaluation) on a clean glass slide under the microscope (10X) with biotherm stage attached. The motility was observed under high power at 40X magnification and expressed in terms of percentage of progressively (0-100) motile sperms.

Viability

To ascertain the percentage of live spermatozoa, 1 drop semen mixed with 2 drops of Eosin-nigrosin (Eosin 1.67 gm, Nigrosin 10 gm, Distilled water 100 ml) was examined under high power (100X) objective. Staining solution cover slips, petridishes were kept at 37 ºC before diluting semen with stain to avoid the manual damage to spermatozoa. Smears were prepared within 30 seconds of mixing. All stained and partially stained spermatozoa were considered dead and the unstained spermatozoa as live. The percentage of live spermatozoa was determined by counting at least 200 spermatozoa.

Sperm abnormalities

Rose Bengal stain (3%) was used for counting the percentage of normal, abnormal sperm (Pervage *et al.,* 2009) [16]. Two drops of sodium-citrate buffer were placed on a clean dry glass slide; one drop of mixed semen was added and spreaded by covering with another slide. The slide was dried in the air and stained with rosebengal stain for 15-20 minutes, rinsed of excess stain by dipping the slide in distilled water. The slide with smear was dried in the air and observed under microscope in high magnifications (100X).

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

The data on motility, viability and abnormal spermatozoa preserved in Group 1, 2, 3 and 4 for three days at 5°C are presented in Table 1.

Different microscopic examination parameters of Deccani ram semen diluted with Tris citrate egg yolk added with three antioxidants at 24 hrs (5 ºC).

There is significant ($P\leq0.05$) difference between control and group I which differ significantly with that of group II and III. While, there was no significant difference ($P\geq0.05$) between group II and III pertaining to individual motility and live spermatozoa (5 ºC) at 24 hrs. There is a significance ($P\leq0.05$) difference in between control and group II and there is no significance difference between group I and III when compared with control and group II pertaining to sperm abnormalities (Table No. 1)

Table 1: There is a significance ($P\leq0.05$) difference in between control and group II and there is no significance difference between group I and III when compared with control and group II pertaining to sperm abnormalities

Particulars	Control	Ascorbic acid (Group 1)	BHT (Group 2)	Vitamin E (Group 3)
Individual Motility (%)	64.983 ± 1.71^{a}	71.983 ± 0.70^{b}	79.250 ± 0.66^{c}	81.566 ± 1.35^{c}
Live spermatozoa (%)	69.090 ± 1.45^{a}	75.090 ± 1.06^{b}	81.796 ± 0.33^{c}	80.063 ± 0.17^{c}
Sperm Abnormalities (%)	6.43 ± 0.16^{b}	6.22 ± 0.11^{ab}	5.96 ± 0.09^{a}	6.19 ± 0.06^{ab}

Means with different superscripts (a, b and c) in the row differ significantly ($P<0.05$).

Different microscopic examination parameters of Deccani ram semen diluted with Tris citrate egg yolk added with three antioxidants at 48 hrs (5 ºC).

There is significant ($P\leq0.05$) difference between control and group I which differ significantly with that of group II and III. While there was no significant difference ($P\geq0.05$) between group II and III pertaining to individual motility and live spermatozoa (5 ºC) at 48 h. The sperm individual motility decreased significantly ($P\leq0.05$) as the period of storage increased from time of dilution to 48 hrs of storage at 5 ºC. There is a significance ($P\leq0.05$) difference in between control and group II and there is no significance difference between group I and III when compared with control and group II pertaining to sperm abnormalities (Table No.2)

Table 2. There is a significance ($P\leq0.05$) difference in between control and group II and there is no significance difference between group I and III when compared with control and group II pertaining to sperm abnormalities

Particulars	Control	Ascorbic acid (Group 1)	BHT (Group 2)	Vitamin E (Group 3)
Individual Motility (%)	53.716 ± 1.46^{a}	60.550 ± 0.69^{b}	70.250 ± 1.06^{c}	68.450 ± 1.00^{c}
Live spermatozoa (%)	58.233 ± 2.07^{a}	65.266 ± 1.76^{b}	72.700 ± 1.67^{c}	71.100 ± 1.69^{c}
Sperm Abnormalities (%)	7.27 ± 0.14^{b}	6.99 ± 0.12^{ab}	6.7 ± 0.10^{a}	6.91 ± 0.10^{ab}

Means with different superscripts (a, b and c) in the row differ significantly ($P<0.05$)

Different microscopic examination parameters of Deccani ram semen diluted with Tris citrate egg yolk added with three antioxidants at 72 hrs (5 ºC).

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 individual motility and live spermatozoa (5 ºC) at 72 h. There was no significant ($P \geq 0.05$) difference between Group I, II and III which differ significantly ($P \leq 0.05$) with that of control. Pertaining to sperm abnormalities (5 ºC) at 72 hrs (Table No.3)

Table 3: There was no significant ($P \geq 0.05$) difference between Group I, II and III which differ significantly ($P \leq 0.05$) with that of control. Pertaining to sperm abnormalities (5 ºC) at 72 hrs

Particulars	Control	Ascorbic acid (Group 1)	BHT (Group 2)	Vitamin E (Group 3)
Individual Motility (%)	42.383±1.61[a]	49.833±0.75[b]	61.250±1.25[c]	58.200±0.93[c]
Live spermatozoa (%)	46.266±0.59[a]	52.833±0.40[b]	61.933±0.84[c]	59.933±0.71[d]
Sperm Abnormalities (%)	8.39 ± 0.10[b]	7.77± 0.13[a]	7.45 ± 0.09[a]	7.78±0.11[a]

Means with different superscripts (a, b and c) in the row differ significantly ($P<0.05$)

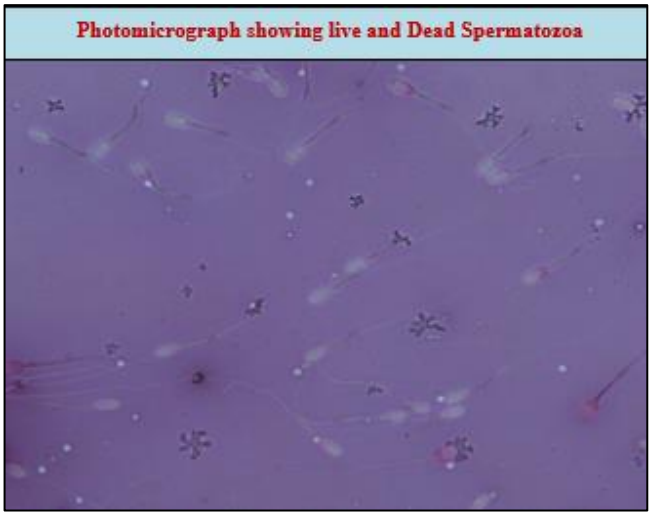

Fig 1: Photomicrograph showing live and dead Spermatozoa

Fig 2: Sperm abnormalities showing dag defect

Discussion

Motility

The observations on individual motility are in accordance with the Anghel *et al*., (2009) noticed that vitamin E had a protective effect over motility. Azawi and Hussein (2013) [4] and Kheradmand *et al*., (2006) [12] reported that addition of antioxidant such as vitamin E to semen preservation media could improve motility in Awassi ram semen.

Findings of the present study are in agreement with Bhakat *et al*., (2006), who reported significant improvement of motility of spermatozoa preserved in Tris based extender fortified with BHT on storage at refrigerator temperature (4 to 7 °C). Similarly extenders containing antioxidant BHT maintained higher sperm motility up to 72h preservation at 4°C than the control and extender with ascorbic acid (Rather *et al*., 2016) [17]. On the contrary, Balla *et al*., (2001) [5] recorded no significant improvement in the maintenance of motility.

Azawi and Hussein, (2013) [4] reported that addition of antioxidant such as vitamin C to semen preservation media could improve motility in Awassi ram semen But Kheradmand *et al*., (2006) [12] addition of ascorbic acid (0.9mg/ml) did not improve the motility in compare with the control group. Rather *et al*., (2016) [17] observed a sudden decline in sperm motility immediately after incorporation of ascorbic acid.

Viability

In this study results are in accordance with Bhakat *et al*., (2011) [7] viability count was significantly higher in Vit E group as compared to the control group. Anghel *et al*., (2009) noticed that 1mM vitamin E had protective effect over viability. There were significant major effects of vitamin E addition to semen diluents on sperm viability in different times of preservation at 5 ˚C ($p<0.05$) than the control in Awassi rams (Azawi and Hussein, 2013) [4]. In contrast, Ball *et al*., (2001) [5], viability is not significant influenced by vitamin E adding in stallion semen after refrigeration for 72 or 96 hrs. The observations of viability are in accordance with the studies of Bhakat *et al*., (2011) [7]. The viability count was significantly ($P<0.05$) higher in BHT group as compared to the control group. Contradict to our study Rather *et al*., (2016) [17] has reported that during the entire period (up to 72 hrs) of preservation, the percent live did not differ significantly ($P>0.05$) amongst the antioxidant incorporated and control groups.

Whereas Azawi and Hussein, (2013) [4] recorded that there was no significant major effect of vitamins C addition to semen diluents on sperm viability in different times of preservation at 5 ˚C than the control in Awassi rams.

Non significant results in stallion semen might be due to more fragile nature of spermatozoa in equines when compared with ovines.

Sperm Abnormalities

These results are in accordance with the studies of Azawi and Hussein (2013) [4], addition of vitamin E resulted in a significant decrease in sperm abnormalities in Awassi ram semen after 120 hr of incubation at 5 ˚C and Bhakat *et al*., (2011) [7] reported that the abnormalities was significantly less in Vit E than the control

Similarly in BHT the results are in accordance with the findings of Bhakat *et al*., (2011) [7] and Rather *et al*., (2016) [17] where the percentages of morphological abnormalities were significantly lower in BHT incorporated group compared to either control or ascorbic acid group at 48 and 72 hrs of preservation.

Similarly in ascorbic acid the results are in accordance with the findings of Azawi and Hussein, (2013) [4] where Addition of vitamin C resulted in a significant decrease in sperm abnormalities of Awassi ram semen after 120 hrs of incubation at 5 ˚C.

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