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Assessment of antioxidants for preservation of crossbred bull semen in Tris based extender

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ABSTRACT

Present study was aimed to improve poor quality crossbred bulls semen, as occurrence of poor quality ejaculates are one of the major problems in crossbred bulls. Antioxidants and its combination was tried to overcome such problems. Vitamin E, Vitamin C and Vitamin E+C were supplemented at dose rate of 1mg, 5 mM and 1mg + 5mM per ml respectively in split ejaculates to evaluate semen quality during preservation at refrigerated temperature and cryopreservation. The results showed that semen characteristics were better in antioxidant supplemented ejaculates. Total sperm abnormality were significantly lower ($p<0.05$) in antioxidant supplemented group. The seminal characteristics of crossbred bull semen showed significantly better ($p<0.05$) performance on preservation, when fortified with Vitamin E as compared to Vitamin C alone as well as Vitamin E and C in combination. The performance of semen additives, were supportive to semen characteristics especially for Vitamin E, moreover fortification with Vitamin E+C was slightly superior to Vitamin C. It can be concluded that fortification of Vitamin E has beneficial role in semen quality improvement followed by Vitamin E+C and Vitamin C.

Key words: Crossbred bull, Vitamin E, Vitamin C, Semen quality.

INTRODUCTION

Crossbreeding programme has played significant role in attaining India's top position in milk production. Out of total milk produced by cow 21% is produced by 56.6 million indigenous cow and rest 19% is produced only by 16.1 million crossbred cows. In other words 22.1% of crossbred cattle produced 48% of total cow milk (Singh, 2016). four to six percent increase in milk production per year is achieved primarily by mating high quality bull with local cows (Kurien, 1987). Artificial insemination and cryopreservation of crossbred bull semen further hasten improvement in milk production. Tris-based extender showed better preservability of bovine semen as compared to plant based soy-extender due to low cost and good *in-vivo* fertility result (Chaudhari *et al.,* 2015). Cryopreservation protocol for semen involves several cascades of steps in series and each step can damage plasma membrane and sperm function (Gadea *et al*., 2004). Moreover, lipid peroxidation due to oxidative damage leads to structural alterations in the acrosomal region of the sperm cell (Bansal and Bilaspuri, 2010). This event due to high concentration of unsaturated fatty acids in membrane further deteriorates sperm characteristics. The limited antioxidant capacity of spermatozoa and ability to generate reactive oxygen species (ROS) decreases the sperm viability (Storey, 1997). Spermatozoa are capable of generating ROS (Balla *et al*., 2001), which is essential for normal physiology and sperm-oocyte fusion. Increase ROS production by weakening spermatozoa can have detrimental effect on sperm function (Baumber *et al*., 2000). Decrease in ATP levels due to oxidative stress reduces sperm motility and fertilizing potential of spermatozoa (Verma and Kanwar, 1998). Moreover, freeze-thaw cycle reduces the level of antioxidant in mammalian semen (Stradaioli *et al*., 2007) and 2/3rd decrease in glutathione level as well. Addition of antioxidant might be helpful in preventing the damage under such condition (Andrabi, 2009; Bhakat *et al.*, 2011). It provide defense at three levels oxidation i.e., prevention, interception and repair (Sies, 1993). To protect the sperm damage extra antioxidant fortification is also advocated (Andrabi *et al.*, 2008). ROS production cannot be eliminated but can be modulated to improve fertility of males by incorporating antioxidants preservative. Vitamin E and Vitamin C are non enzymatic antioxidants. The most important antioxidants in seminal fluid seem to be Vitamin C and E (Niki, 1991). The Vitamin C in seminal plasma is 10 times i.e., 364 µmol L^{-1} as compared to blood plasma (40 µmol L^{-1}). Vitamin E acts as a lipid soluble antioxidant in cell membranes which effectively control lipid peroxidation and reduce DNA damage. Due to its lipid solubility, Vitamin E is the first line of defense against the peroxidation of PUFA (Poly unsaturated fatty acid) in cellular membranes. Vitamin E is key chain contra-veining antioxidants in membranes which directly trap the free radicals such as superoxide anions,

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hydrogen peroxides and hydroxyl radicals (Balla *et al*., 2001). Vitamin E has significant effect with respect to frozen semen characteristics however its effect under refrigerated condition was not up to the mark (Shukla and Misra, 2005). Literature on combination antioxidant fortification was scanty.

Therefore, present investigation was undertaken to compare the effect of antioxidants, such as Vitamin E, Vitamin C and Vitamin E+C (Vitamin E and Vitamin C in combination) on seminal characteristics of Karan-Fries [Holstein-Friesian (HF)-Tharparkar cross] bulls in Tris based extender.

MATERIALS AND METHODS

The present investigation was conducted on 20 poor grade (≤+3.00 mass activity in 0-5 scale) ejaculates to identify best additive at different hours of preservation (0, 24 and 48) at refrigerated (5°C) temperature and 15 high grade (> 3.5 mass activity) ejaculates subjected to cryopreservation from 5 KF bulls maintained at Artificial breeding Research Center NDRI, Karnal. The semen was collected once in a week with two successive ejaculations from each bulls using sterilized artificial vagina. Pooled ejaculate was splitted into four parts for dilution with Tris egg yolk extender along with different antioxidants i.e. i. No antioxidant (control), ii. Vitamin E @1mg/ml iii. Vitamin C @ 5mM/ml and iv. Vitamin E+C @ 1mg + 5Mm/ml at both refrigeration at 5°C and cryopreservation for further analysis. Semen was analysed for motility, non eosinophilic count, sperm abnormality, intact acrosome (Andrabi *et al.*, 2008) and plasma membrane integrity by hypo osmotic swelling test (Jeyendran *et al.*, 1984) at 0, 24 and 48 hours of refrigeration as well as pre-freeze and post-thaw stages for cryopreserved semen. Semen samples were extended in Tris-citric acid-Fructose egg yolk extender with glycerol @6.9% and added with antioxidants. Semen was cooled slowly up to 5°C and equilibrated for 4 hours after packing in 0.25ml polyvinyl French straw. Straw were then placed horizontally on a rack and frozen in vapour 4 cm above liquid nitrogen (LN) for 10 minutes then dipped into liquid nitrogen. Frozen straw were thawed at 37°C for one minute. Data were analysed using ANOVA technique (Snedecor and Cochran, 1994) and prior to the analysis proportionality data (motility, per cent non-eosinophilic count, HOST, intact acrosome and abnormality) were transformed using the arcsine transformation [asin (sqrt (percent/100))] with adjustment to allow for zero values. Comparison between different treatment groups were done by Duncan multiple range test (DMRT). The differences at $p \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The quality of semen was assessed for both preservation at 5°C and -196°C and the result were presented in Table-1 and 2, respectively.

Performance of additives at refrigerated temperature (5°C): Sperm motility at 0 hrs was higher in additives group compared to control but no significant difference was observed; at 24 hrs motility was significantly ($p<0.05$) higher in supplemented group as compared to control, moreover motility in Vitamin E aided sample was significantly ($p<0.05$) higher as compared to vitamin E+C. At 48 hrs of incubation, additives group motility was significantly ($p<0.05$) higher compared to control and within additives group motility in Vitamin E was significantly ($p<0.05$) higher compared to Vitamin C and E+C, however motility in vitamin C and E+C didn't differ significantly. Average HOST (%) at '0 hrs' was significantly ($p<0.05$) higher in vitamin E as compared to the control; at 24 and 48 hrs of incubation, supplemented

Table 1: Seminal Characteristic (%) in refrigerated semen (N=20) preserved with antioxidants in Tris- extender:

Duration of storage	Seminal Characteristics	Control	Vitamin E @ 1mg/ml	Vitamin C @ 5mM/ml	Vitamin E+C @ 1mg + 5mM/ml
0 hrs	**IM**	52.46±0.01	55.42±0.01	54.52±0.01	54.41±0.01
	HOST	54.27^{a}±0.01	58.64^{b}±0.01	56.27±0.01	56.07±0.01
	N-Eosin	63.75^{a}±0.01	68.12^{b}±0.01	65.75±0.03	65.66±0.02
	IA	68.48^{a}±0.01	73.54^{b}±0.02	70.22^{a}±0.02	70.52^{a}±0.03
	TA	26.20^{a}±0.01	20.69^{b}±0.03	23.67^{c}±0.01	21.52^{b}±0.01
24 hrs	**IM**	35.89^{a}±0.04	43.76^{b}±0.03	41.11^{cb}±0.02	40.47^{c}±0.05
	HOST	37.04^{a}±0.02	45.47^{b}±0.03	42.41^{cb}±0.03	41.36^{c}±0.02
	N-Eosin	47.23^{a}±0.03	55.53^{b}±0.04	52.10^{c}±0.02	51.25±0.01
	IA	52.91^{a}±0.02	61.75^{b}±0.01	58.87^{c}±0.03	57.62^{c}±0.02
	TA	44.60^{a}±0.01	22.36^{b}±0.03	26.66^{c}±0.01	26.51^{c}±0.03
48 hrs	**IM**	11.98^{a}±0.07	19.77^{b}±0.03	16.07^{c}±0.02	16.23^{c}±0.01
	HOST	14.82^{a}±0.03	22.60^{b}±0.02	18.86^{c}±0.03	19.18±0.04
	N-Eosin	19.50^{a}±0.03	28.01^{b}±0.03	23.71^{c}±0.02	23.99^{c}±0.03
	IA	23.35^{a}±0.04	35.41^{b}±0.03	31.52^{c}±0.02	31.67^{c}±0.02
	TA	49.10^{a}±0.02	29.36^{b}±0.01	33.57^{c}±0.01	33.41^{c}±0.02

[Means bearing different superscript within same row differ significantly ($^{abc}p<0.05$)]

IM: Individual motility (%), **HOST:** Hypo-osmotic swelling test reactivity (%), **N-Eosin:** Non-eosinophilic count (%), **IA:** Intact acrosome (%), **TA:** Total abnormalities (%) of sperm.

Table 2: Seminal Characteristic (%) in pre freez and post thaw semen (N=15) preserved with different antioxidants in Tris- extender:

StorageCondition	Seminal Characteristics	Control	Vitamin E @ 1mg/ml	Vitamin C @ 5mM/ml	Vitamin E+C @ 1mg + 5mM/ml
Pre Freez	**IM**	76.88±0.02	81.09±0.01	78.05±0.02	79.12±0.02
	HOST	72.54±0.01	76.33±0.01	74.36±0.01	68.98±0.16
	N-Eosin	81.93±0.02	85.92±0.01	83.41±0.02	83.82±0.03
	IA	85.77^{a}±0.01	89.55^{b}±0.02	86.74^{a}±0.01	87.52±0.01
	TA	7.28^{ac}±0.02	6.10^{b}±0.01	6.78^{c}±0.03	6.65±0.01
Post Thaw	**IM**	43.75^{a}±0.01	50.69^{b}±0.02	46.83^{ca}±0.01	47.14^{c}±0.02
	HOST	38.73^{a}±0.01	45.83^{b}±0.01	42.27^{c}±0.02	42.35^{cb}±0.01
	N-Eosin	48.99^{a}±0.01	56.01^{b}±0.01	51.92^{a}±0.02	52.01^{a}±0.01
	IA	56.24^{a}±0.01	63.57^{b}±0.01	59.48^{c}±0.02	59.72^{c}±0.01
	TA	18.36^{a}±0.04	16.02^{b}±0.02	17.62^{c}±0.01	17.36^{c}±0.01
Post Thaw	% improvement of motility	-	15.86	7.04	7.74

[Means bearing different superscript within same row differ significantly ($^{abc}p<0.05$)]

IM: Individual motility (%), **HOST:** Hypo-osmotic swelling Test (%), **N-Eosin:** Non-eosinophilic count (%), **IA:** Intact acrosome (%), **TA:** Total sperm abnormalities (%).

group HOST was significantly ($p<0.05$) higher as compared to the control group. Among additives groups, Vitamin E aided sample was significantly ($p<0.05$) higher in HOST as compared to the Vitamin C and E+C, but there was no significant difference between in Vitamin C and E+C treated group. In similar line, Batool *et al.* (2012) reported Vitamin E (0.5m/M) in extender improved plasma membrane functionality, but dose of Vitamin E was lower than our supplementation. The Non eosinophilic sperm count and intact acrosome (IA) showed similar trends as HOST at 0, 24 and 48 hrs of preservation. The positive result of antioxidants fortification might be due to its ability of scavenging free radicals, which are associated with diluents and sperm both to minimize stress on motility device of sperm including plasma membrane (Pena *et al*., 2003). Addition of Vitamin E decreases lipid peroxidation as observed by rise in thiobarbutric acid reactive substance (TBARS) production in pig (Cerolini *et al*., 2000). In our study total abnormality (TA) was found to be least in extender fortified with Vitamin E followed by Vitamin E+C and Vitamin C, respectively at refrigerated temperature. In Vitamin E+C aided group tail and total abnormality was lower than vitamin C, it might be due to some complementary effect of Vitamin E and C. Abnormal sperm morphology has been correlated with reduced viability and fertility in cattle (Sajjad *et al.*, 2007). Additionally, the morphology and sperm activity would be preserved by the binding of Vitamin E to endoperoxides (Luvoni *et al*., 2006). Superiority of Vitamin E+C over Vitamin E and C alone was reported by Mittal et al. (2014), but with different dose rate in buffalo bull smen. Similar to our finding ascorbic acid plays a role in protecting sperm from ROS was also reported earlier (Buetter, 1993) and maintaining the integrity of sperm cells by preventing oxidative damage to sperm DNA (Fraga *et al.*, 1991) as lipid peroxidation begins at the time of ejaculation rather during semen storage (Blesboi *et al*., 2005). Antioxidant neutralizes oxidizable ingredients therefore, significantly delay or reduce oxidation of substrate at low concentration. Membrane fluidity present in sperm is due to high PUFA which is susceptible to lipid peroxidation. Membrane fluidity plays a significant role in calcium pump regulated influx and out-fluxes of calcium ion into the spermatozoa. Alteration in membrane fluidity causes accumulation of calcium ion in membrane which damage sperm motility and viability (Saleh and Agarwal, 2002).

Performance of additives at ultra low temperature (-196°C): A comparative study of performance of various additives at freezing temperature (-196°C) was carried out and the data are shown in (Table-2).

At pre-freez stage, least squares means of individual motility, Non-Eosinophilic count and HOST was higher in additives group as compared to control, but it was not significant moreover within fortified group. IA was significantly ($p<0.05$) higher in Vitamin E and C supplemented group and TA was significantly ($p<0.05$) lower in Vitamin fortified group. At post-thaw stage individual motility (percent) was significantly ($p<0.05$) higher in fortified group as compared to control except Vitamin C. Non-Eosinophilic count, HOST and IA were significantly ($p<0.05$) higher and TA was significantly ($p<0.05$) lower in antioxidant fortified group. Percent improvement in motility over control at '0 hrs' post thaw was 15.86, 7.04 and 7.74 for Vitamin E, Vitamin C and Vitamin E+C, respectively. Similarly, in a finding (Andrabi *et al.*, 2008) on buffalo semen higher post-thaw motility, plasma membrane integrity and normal apical ridge was reported after addition of ascorbic acid and á-tocopherol in tris based extender. Herdis *et al.* (2002) reported improvement in sperm quality with respect to post-thaw live percentage (75 Vs 64.8%) and membrane intact percentage (65.8 Vs 55.2%) in case of α-tocopherol addition as compared to control. Vitamin C is capable of neutralizing H_2O_2 produced in a hydrophilic environment and can protect membrane integrity of sperm cells from heat

shock during dilution-cooling and storage process of spermatozoa (Lewis *et al*., 1997). However, supplementation of antioxidant in milk based extender did not improve semen quality (Akhter *et al*., 2011) might be due to naturally occurring antioxidant casein in milk. Semen characteristics was found to be preserved better in extender fortified with additives, however among additives Vitamin E was superior followed by Vitamin E+C and Vitamin C respectively in Tris-based extender in the present study. Best effect of Vitamin E as preservative might be due to its chemical nature (lipid soluble) and permeability through the plasma membrane of spermatozoa and additional antioxidant concentration system. Similar to present finding superiority of Vitamin E over Vitamin C especially with reference to HOST percent was also reported (Andrabi *et al.*, 2008; Swain and Kundu, 2009). Vitamin E added at different rate improved sperm profile in other animals also like Ghazel (Pour *et al*., 2013), equine (Balla *et al*., 2001), sheep or goat (Hartono, 2008) and buffaloes (Beheshti, 2011). In similar line dose of Vitamin E 0.1 to 1.0 mM in extended semen showed improved semen characteristics and cytological parameters in ram semen (Anghel *et al*., 2000). More positive effect on semen quality and preservability is detected with poor quality semen (Pour *et al*., 2013). Significant positive effect was also reported in chicken semen fortified with Vitamin E at the rate of 2% (Tabatabaei *et al*., 2011). The beneficial effect of Vitamin E on sperm motility was previously also observed (Boonsorn *et al*., 2010). Vitamin E in higher concentration may be act as oxidation stimulator rather than antioxidant (Breininger *et al*., 2005). Addition of Vitamin C resulted in slight improvement in semen profile (Michael *et al.*, 2007), supports our finding. Ascorbic acid is essential cofactor for at least eight enzymes and can also act as an antioxidant by reacting with free radicals. However, the little improvement in motility (Sonmez and Demirci, 2004) could be due to lower dose of Vitamin C use. Most animals can synthesize ascorbic acid from glucose through glucuronic acid pathway. Ascorbic acid is required as a cofactor for more than eight enzymes (Halliwell and Gutteridge, 1999). Presence of metal ions like Fe^{3+}, Cu^{2+} with high concentration of ascorbic acid can act as pro-oxidants. Superiority of Vitamin E over Vitamin C was reported is in agreement with finding of other investigators with similar dose regime used as in present study (Shukla and Misra, 2005; Azawi and Hussein, 2013).

CONCLUSION

A large gap is observed between supply and demand of semen due to poor quality sperm especially in crossbred bulls.There is need of improvement in the quality of semen especially of low grade type to fill the gap of demand under field condition. Enrichment of antioxidant capacity of semen can be very good opportunity for improving fertility. When poor quality semen was fortified with additives (Vitamin E, Vitamin C and Vitamin E+C), there is additional beneficial effect in terms of semen quality especially short and long term storage of spermatozoa. This approach of additional antioxidant fortification in existing antioxidant system may be useful to improve fertilizing potential of low quality ejaculated which is usually discarded by one or other way. Therefore, present module of antioxidant fortification can be practiced in existing semen handling protocol at semen station to overcome the semen deficit.

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