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Improvement in quality of frozen Jersey bull semen following fortification with chloroquine diphosphate and ascorbic acid

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ABSTRACT

The present study was conducted with an objective of studying the role of certain additives e.g. chloroquine diphosphate and ascorbic acid in maintaining the membrane integrity, viability and motility of spermatozoa at various pre- and post-freezing stages of semen of Jersey bulls maintained under sub-temperate climate. Work was conducted at Sperm Station Palampur, Himachal Pradesh, India, on six apparently healthy pure bred Jersey breeding bulls. A total of 36 ejaculates (6 from each bull), were analysed at six different stages of processing namely neat semen evaluation, post-dilution, post-equilibration, post-thaw and after 1 and 2 h incubation post-thaw at 37°C for progressive motility, live dead count, reaction to hypo-osmotic solution and acrosomal integrity, respectively. Evaluation of the semen was done for three groups, i.e. control group (G_1), semen fortified with chloroquine diphosphate (G_2) and ascorbic acid (G_3).The results of the study revealed a significant improvement in progressive motility, live sperm percentage, reaction to hypo-osmotic solution and acrosomal integrity of semen having chloroquine diphosphate (G_2) and ascorbic acid (G_3) as additives when compared to control group. So, fortification of semen should be done with chloroquine diphosphate and ascorbic acid to improve the quality of semen in future.

Key words: Ascorbic acid, Chloroquine diphosphate, Jersey bulls, Live sperm percentage, Progressive motility

In the present scenario of spiraling human population growth, there is an increased pressure for augmenting milk production as per equation of demand and supply. Mammalian spermatozoa are extremely sensitive to oxidative damage, *in-vivo* as well as *in-vitro*. Lipid peroxidation plays a leading role in spermatozoon aging, shortening its life span *in-vitro* and affecting the preservation of semen for artificial insemination. Cold shock treatment also causes the spermatozoon to increase its susceptibility to lipid peroxidation. Therefore, to protect the spermatozoa from physical and physiological damages, the role of membrane stabilizer(s) and natural antioxidant(s) addition comes into play, as it helps to improve the quality of cryopreserved semen. The presence of high concentrations of long chain polyunsaturated fatty acids within the lipid structure of sperm cells requires efficient antioxidant systems to defend peroxidative damages associated with sperm dysfunction (Cecil and Baskt 1993). Long term storage and preservation of animal semen remains a subject of interest ever since artificial insemination (AI) embarked on a large scale. The success of cryopreservation depends largely on the specific susceptibility of sperm cells to low temperature (Sharma *et al.* 2012).

Attempts to reduce peroxidative damage during semen cryopreservation have also been made by adding ascorbic acid in the semen diluents. Ascorbic acid, a biologically active reducing agent, has been demonstrated to restore fertility possibly by reduction of anti-agglutination factor on sperm membrane from inactive form to active form (Lindahl 1966). Membrane stabilizers and antioxidants have beneficial effect on the membrane integrity and biofunctional activity of spermatozoa. The beneficial effect of chloroquine diphosphate as membrane stabilizer in preservation of buffalo semen has been demonstrated by different workers (Kumar 1992). The present investigation was undertaken to study the effect of certain semen diluents additives (chloroquine diphosphate and ascorbic acid) on the preservability of Jersey bull semen *in-vitro* vis-a-vis *in-vivo*.

MATERIALS AND METHODS

The study was conducted on six apparently healthy pure bred Jersey breeding bulls, aged between 5–8 years, maintained at Sperm Station, Palampur, Himachal Pradesh, India (32.6°N, 76.3°E, altitude 1290.8 m). A total of 36 ejaculates (6 from each bull) were investigated during October 2017 in the present study. Semen was collected twice a week from each bull. Bulls were already trained to donate semen in the artificial vagina.

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Semen evaluation and processing

Neat semen: Collected semen was examined for mass motility, progressive motility, live and dead count of spermatozoa, acrosomal integrity and hypo-osmotic swelling test (HOST) before processing.

Semen dilution and evaluation: After evaluation, neat semen was extended in Tris based extender (Tris-2.42 g, Citric acid monohydrate-1.36 g, Glucose-1.00 g; Sigma-Aldrich Corporation) to a concentration of 60 million/ml and extended semen was divided into three aliquots. First aliquot (10 ml diluted semen) served as control (G_1), while other two were modified either with chloroquine diphosphate (G_2) or ascorbic acid (G_3) for having desired concentration of additives.The final concentrations of chloroquine diphosphate and ascorbic acid were 10^{-5} M and 0.02% in the G_2 and G_3 groups, respectively. Finally diluted semen (control) and treated samples placed in the water jugs (37°C) were shifted to the cooling cabinet. All three groups were again subjected to the evaluation tests viz. progressive motility, live dead count, acrosomal integrity and hypo-osmotic swelling test (Sharma *et al.* 2012)

Equilibration, filling, sealing and pre-freezing evaluation
Freezing of the semen

Thawing of semen and post-thaw incubation evaluation: Semen straws from all three groups were thawed at 37°C for 30 sec and semen samples were evaluated at 0, 1 and 2 h post-thaw for progressive motility, differential staining of live dead spermatozoa, acrosomal characteristics and for reaction to hypo-osmotic solution i.e. HOS test.

Statistical analysis: The data were analyzed by ANOVA with SPSS® 20 level version for windows.

RESULTS AND DISCUSSION

It is very useful to estimate the percentage of live spermatozoa in the semen sample in addition to other parameters, since some live non-motile spermatozoa become motile after dilution and storage (Tomar *et al.* 1969) and only the proportion of viable spermatozoa are able to participate in vital processes in reproduction.

Viability: The mean live sperms percentage after extension with Tris dilutor for G_1, G_2 and G_3 was 80.53±0.76, 81.17±0.83 and 80.49±0.82, respectively, indicating that practically there was no difference among groups (Table 1). This viability may be related to those reported by Verma (1997). After equilibration, live sperms percentage was 76.77±0.68 for untreated semen (G_1) which was nearly in accordance with Nath *et al.* (1996) and higher than that reported by Angasaria *et al.* (2002). These differences may be due to the cooling and equilibration time and glycerolization (Veeraiah *et al.* 1999). A combination of livability along with post-thaw motility is a good index for judging semen quality and probably fertility status of bull (Singh and Pant 2001). Present viability and motility was higher than those reported by Angasaria *et al.* (2002) and Shanmugavel and Singh (2002), and lower than those recorded by Singh and Pant (2001). This variation could be due to thawing procedure and glycerol concentration (Robbins *et al.* 1976), freezing rate and time of equilibration, and age of egg used in diluent (Veeraiah *et al.* 1997).

It is evident that after freeze-thawing there was certain damage to the cytoskeleton of spermatozoa and hence livability of spermatozoa was reduced. Effect of additives in the dilutor on post-thaw (0 h) livability was encouraging and an increase in percent live sperm was noticed (Table 1). It is evident that the progressive fall in live sperms per hour was slightly less (8.44%) in ascorbic acid group and provided better protection than chloroquine diphosphate (8.89%). Increase in post-thaw livability in presence of ascorbic acid and chloroquine diphosphate has been reported (Singh 2002). The better effect of ascorbic acid as compared to chloroquine diphosphate may be because of less damage in the presence of ascorbic acid as it has been reported to protect the sperm cell from peroxidase damage (Sidhu *et al.* 1996).

Motility: The estimate of mass motility is not very precise to judge the individual sperm motility.Addition of additives improved the progressively motile sperm percentage marginally but the difference was non-significant (Table 1). However, Singh (2002) and Kumar (2007) recorded better motility in additives (ascorbic acid and chloroquine diphosphate) added semen at this stage in buffalo semen and this variation could be due to species. After freeze-thawing, post-thaw motility reduced and the marked reduction in sperm motility could be attributed to death of spermatozoa during freezing, reduction of cAMP (Kakar and Anand 1984), and decrease in ATP level due to

Table 1. Effect of additives on live sperm and progressive motility (%) in semen of Jersey bulls

Stage of semen processing	G_1 (Control group)		G_2 (Chloroquine diphosphate)		G_3 (Ascorbic acid)	
	Live sperms	Progressive motility	Live sperms	Progressive motility	Live sperms	Progressive motility
Neat semen	81.39±0.88	75.42±0.55	81.39±0.88	75.42±0.55	81.39±0.88	75.42±0.55
After dilution at 37°C	80.53±0.76	74.31±0.41	81.17±0.83	74.97±0.38	80.49±0.82	74.14±0.39
Post equilibration at 4°C	76.77±0.68	71.06±0.65	78.64±0.78	72.36±0.57	78.86±0.76	71.61±0.52
Post-thaw (0 h)	53.16±1.19^{a}	47.58±1.08^{a}	56.58±1.14^{ab}	50.14±1.01^{ab}	57.39±1.02^{b}	51.28±0.90^{b}
1 h post-thaw incubation	44.50±1.16^{a}	38.69±0.92^{a}	47.39±1.19^{ab}	40.97±1.02^{ab}	47.55±1.07^{b}	41.86±0.82^{b}
2 h post-thaw incubation	36.22±0.08^{a}	28.28±0.98^{a}	38.80±1.13^{ab}	32.03±1.04^{b}	40.50±0.96^{b}	32.67±0.90^{b}

Means with different superscripts within the same row differ significantly for the same parameter ($P<0.05$)

Table 2. Effect of additives on HOS and acrosomal integrity (%) in semen of Jersey bulls

Stage of semen processing	G_1 (Control group)		G_2 (Chloroquine diphosphate)		G_3 (Ascorbic acid)	
	HOS reactive	Acrosomal integrity	HOS reactive	Acrosomal integrity	HOS reactive	Acrosomal integrity
Neat semen	66.42±1.12	87.28±0.94	66.42±1.12	87.28±0.94	66.42±1.12	87.28±0.94
After dilution at 37°C	67.47±0.05	86.17±0.94	68.97±1.14	86.94±0.80	69.17±0.92	86.89±0.92
Post equilibration at 4°C	63.31±1.11	82.47±1.05	64.97±0.97	84.06±0.82	64.58±0.98	84.47±0.86
Post-thaw (0 h)	40.78±0.93[a]	67.92±0.93	45.97±0.76[b]	68.36±0.79	45.56±0.99[b]	69.97±0.56
1 h post-thaw incubation	33.06±0.83[a]	59.69±0.84[a]	39.22±0.76[b]	62.06±0.73[b]	37.94±0.93[b]	63.47±0.76[b]
2 h post-thaw incubation	25.36±0.77[a]	52.28±0.83[a]	31.22±0.73[b]	56.44±0.98[b]	30.56±0.86[b]	56.72±0.76[b]

Means with different superscripts within the same row differ significantly for the same parameter ($P<0.05$).

inability of mitochondrial enzymes to produce ATP (Heath and Gupta 1980).

The mean post-thaw motility observed in control semen during this study was similar to some previous reports (Veeraiah *et al.* 1999). However, lower (Thakur 2003) and higher (Srivastava and Kumar 2014) progressively motile spermatozoa have also been reported at post-thaw stage. This variation may be due to kind of dilutor (Rao *et al.* 2002) and glycerol concentration (Sagdeo *et al.*1991).

After addition of ascorbic acid (G_3), the significant ($P<0.05$) increase in post-thaw motility was about 3.7% in our study. Such improvement had been reported earlier by Verma and Kanwar (1998). However, Aurich *et al.* (1997) could not find any substantial improvement in post-thaw motility with ascorbic acid treated semen which may be due to the variation in the concentration of ascorbic acid used. Better protection provided by the ascorbic acid serves an evidence of its anti-oxidative property which might be due to the fact that cryopreservation and thawing of bovine spermatozoa in egg yolk-Tris-glycerol extender reduces sperm GSH levels by 78% and SOD activity by 50% (Srivastava and Kumar 2014).

Hypo-osmotic swelling test: Deterioration of spermatozoa function due to change in structural components occurs during the process of semen processing, freezing, cryostorage and thawing (Centola *et al.* 1992). Hence, the study of sperm membrane functional test is of specific importance.

In the present study, significant ($P<0.05$) increase in spermatozoa that reacted to hypo-osmotic solution was found at post-thaw stage in the semen supplemented with ascorbic acid and chloroquine diphosphate and the corresponding vales were 40.78±0.93, 45.97±0.76 and 45.56±0.99 for G_1, G_2 and G_3, respectively (Table 2). These observations were in agreement to that of Singh (2002) and Kumar (2007). The increase in HOS reactive spermatozoa in the presence of additives was also in consonance with the findings of Srivastava (2000) where he recorded difference of 9.44% with ascorbic acid and 4.52% with chloroquine diphosphate added semen.

Significant increase in the post-thaw HOS positive spermatozoa in the presence of ascorbic acid may be due to its effect as an antioxidant that reduces the damage caused by free radical (Srivastava and Kumar 2014) and chloroquine diphosphate (membrane stabilizer) is responsible for protecting the membrane damage to sperm cells. The membrane stabilizing effect of ascorbic acid (Aurich *et al.* 1997) and chloroquine diphosphate had been reported earlier. Chloroquine diphosphate is reported to act as a phosphodiesterase inhibitor which appears to be responsible for enhancing metabolic activity of spermatozoa (Norman and Gombe 1975).

Acrosomal integrity: Acrosome, carrying various enzymes, plays an important role in the process/events of fertilization. Detachment of acrosome or loss of acrosomal membrane integrity may results into decrease ATP and loss of intracellular enzymes and proteins. The assessment of acrosomal integrity is therefore, always a part of assessment of spermatozoa (Noakes *et al.* 2009).

No significant difference among treatment groups was noticed in post-thaw incubated semen. These observations were similar to those reported by Rao *et al.* (2002). Whereas, higher (Thakur 2003) as well as lower (Al-Khanak and Al-Hanak 1989) acrosomal integrity has also been observed. As expected, like other three parameters, acrosomal abnormalities increased following incubation of thawed semen indicating a progressive deterioration of semen quality with post-thaw interval (Table 2). In support to our study, higher percentages than present study have been reported (Singh and Nigam 1998).

Finally, it is evident that the protection for acrosome during post-thaw incubation was higher with ascorbic acid followed by chloroquine diphosphate. Similar results had been reported by Kumar (2007) and Srivastava and Kumar (2014). Ascorbic acid has its beneficial effect on semen freezing through preventing damage caused by reactive oxygen species. Though its effect was not evident in statistical terms immediately after thawing, however it was well noticed following incubation.

In brief, ascorbic acid (anti-oxidant) as well as chloroquine diphosphate (membrane stabilizer) improved the post-thaw quality of frozen semen of Jersey bulls in our study. Also, ascorbic acid gave numerically better (however, non-significant) protection to frozen spermatozoa of Jersey bulls as compared to chloroquine diphosphate. So, a conclusion can be drawn that additives added to the

semen helped in improving the quality of semen.

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