



Impact of supplementation of semen extender with antioxidants on quality of cooled or cryopreserved Marwari stallion spermatozoa

PRASHANT KUMAR¹, ASHOK KUMAR¹, J S MEHTA¹, G N PUROHIT², S K RAVI³, YASH PAL³, R A LEGHA³,
B N TRIPATHI⁴ and TR TALLURI^{3✉}

ICAR-National Research Centre on Equines, Bikaner, Rajasthan 334 001 India

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ABSTRACT

The aim of the present study was to evaluate the effects of supplementation of semen extender with two antioxidants namely Ascorbic acid (AA @ 0.9 g/L), Glutathione (GSH @ 2.5 mM), and combination of both (AA @ 0.9 g/L + GSH @ 2.5 mM) either in alone or in combination on the quality of cooled or cryopreserved Marwari stallion spermatozoa. For this purpose, a total of 24 ejaculates were collected from four adult and fertile Marwari stallions (6 ejaculates from each stallion) using an artificial vagina. Each freshly ejaculated semen sample was investigated for the semen quality parameters, viz. colour, consistency, total volume, gel volume, gel free volume, pH, progressive sperm motility, sperm concentration, sperm viability, sperm plasma membrane integrity, acrosomal integrity and DNA integrity. In the freshly ejaculated semen, no significant variation was found among individual stallions for various semen quality parameters except in sperm concentration. Pre-freeze and post-thaw semen evaluation revealed that the values for the most of the semen quality parameters were significantly higher in the semen extender being treated with the combination (AA @ 0.9 g/L + GSH @ 2.5 mM) of antioxidants group rather than AA and GSH alone or control. Addition of AA (0.9 g/L) and GSH (2.5 mM) to the freezing extender improved equine pre-freeze and post-thaw semen quality with the superiority of control group which indicates the beneficial role of supplementation of antioxidants to the stallion semen during cryopreservation process.

Keywords: Antioxidants, Ascorbic acid, Cryopreservation, Glutathione, ROS, Stallion semen

Cryopreservation of semen has created a new dimension in equine breeding industry by the possible preservation of this biological material for long time. However, compared with other domestic species, the fertility of frozen-thawed stallion sperm remained low (Gibb *et al.* 2017). Cryopreservation of semen imposes some deleterious effects on spermatozoa, either killing a certain proportion of cells or causing damages on sperm function in the remaining surviving population (Yogesh *et al.* 2018). Damages to spermatozoa during the cryopreservation and thawing occur mostly due to oxidative stresses as a result of production of free radicals or excessive reactive oxygen species (ROS) (Papa 2016). Spermatozoa are susceptible to attack by oxidative stress due to deficiency in the levels of intracellular antioxidant enzymes as a result of their low amount of cytoplasm (Bansal and Bilaspuri 2010). To counteract the destructive effects of ROS, seminal plasma has an antioxidant system containing various sperm

oxidative defense enzymes for the protection of sperms. In controlled concentration ROS facilitate, capacitation of sperm, acrosomal reaction and signaling processes to ensure fertilization, implantation and early embryo development. But, the over-production of ROS causes structural damage of sperm membranes (Sakkas *et al.* 1998).

Addition of antioxidants to the seminal plasma or extenders can be used for freezing to improve sperm viability and consequently, fertilizing capacity, by reducing oxidative effects caused by ROS (Zhandi and Ghadimi, 2014, Oliveira *et al.* 2015, Papa 2016). Ascorbic acid (AA) is an effective non-enzymatic antioxidant and water-soluble ROS scavenger with high potency and is potentially involved in protecting cells against oxidative stress. The addition of AA in a semen extender can impact on optimal sperm performance by reducing cell damage through its continuous radical-scavenging action. Glutathione (GSH) is the primary antioxidant present in equine semen and one of the antioxidant added to different semen specimens (Oliveira *et al.* 2013). This thiol has an important role in the anti-oxidation process of endogenous and exogenous compounds, as well as in the maintenance of intracellular redox conditions.

Thus, an alternative to avoid deteriorative negative effect on spermatozoa during cooling and freezing process would

Present address: ¹College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan. ²ICAR-Central Island Agricultural Research Institute (CIARI), Port Blair. ³Equine Production Campus, ICAR-NRC on Equines, Bikaner, Rajasthan. ⁴Deputy Director General (Animal Sciences), Krishi Bhawan, New Delhi. ✉Corresponding author email: raotalluri79@gmail.com

be an antioxidant therapy. Keeping this in view, the present study was conducted to investigate the effect of addition of different antioxidants AA @ 0.9 g/L, GSH @ 2.5 mM and combination of both (AA @ 0.9 g/L + GSH @ 2.5 mM) to semen extender on pre-freeze and post-thaw semen quality parameters and to study comparative efficacy of these antioxidants.

MATERIALS AND METHODS

Experimental animals: Apparently healthy Marwari stallions (4), 4–6 year-old, maintained at a Government Research Organization, Bikaner, Rajasthan were enrolled in the present study. All the experiments were carried out in accordance with the guidelines set out by the Institute Ethics Committee. All the experiments were conducted during breeding season.

Semen collection and processing: The semen samples were collected twice weekly using a colorado model of Artificial Vagina (AV) on estrus mare as dummy. Each stallion was given one false mount before actual collection. The semen samples were collected directly into a clean dry graduated bottle attached to the latex cone of the AV. The tubes containing semen were marked and placed in a water bath at 37°C immediately after collection. Total ejaculate volume was noted as visible from graduated collection bottle, filtered through sterilized gauze and gel free semen volume was noted in another graduated bottle. Gel free semen was mixed with primary extender (Citrates– EDTA extender) in equal and divided in four equal aliquots to centrifuge at 650 g for 3 min. The sperm pellets were extended with secondary semen extender (Glucose– Lactose-EDTA, with Dimethyl formamide (DMF) @ 5% as cryoprotectant having different concentrations of AA and GSH, i.e. 0.9 g/L AA (Group 1), 2.5 mM GSH (Group 2) and 0.9 g/L AA+2.5 mM GSH (Group 3) and control group (Group 4) without any extender respectively. The final sperm concentration (150×10^6 sperms/ml) with secondary extender was achieved. Packaging of extended semen was done using automatic straw filling and sealing machine pre-cooled in a cooling cabinet maintained at 4–5°C for 2 h.

Cryopreservation of stallion semen: The equilibrated semen was loaded into 0.5 ml polyvinyl chloride straws (IMV-Technologies, France), sealed with an automatic filling and sealing machine (IMV-Technologies, L'Aigle, France) and then cooled to 4°C over 2 h as equilibration period. After equilibration, freezing of the stallion semen was performed by customized method of freezing in liquid nitrogen vapours by spreading the straws on a straw stand at height of 4 cm and then the straws were taken out after 12 min exposure and plunged into canisters of liquid nitrogen (–196°C) containers till further analysis. The straws were thawed in a water bath at 37°C for 30 sec immediately before semen analyses.

Assessment of seminal parameters

Sperm motility: A Computer Assisted Semen Analyzer (HTB CEROS II, Version 1.3, Hamilton Thorne Research,

Beverly, MA, USA) equipped with a thermostage (MiniTherm®, Hamilton Thorne Inc. Beverly, MA, USA), was used to analyze the progressive sperm motility. About 4 µl diluted aliquots of semen sample (50 µl of semen sample dissolved in 1 ml of 2.9% sodium citrate diluting fluid to make a 1 : 20 dilution) was loaded in disposable chambers having a 20 µm chamber depth (Leja® Standard Count 4 Chamber Slide, 20 µm, Leja® Products B.V., Netherlands). The HTB CEROS II system was set to measure 30 frames per field at a frame rate of 60 Hz. A minimum of seven fields and 500 spermatozoa were measured for each sample. Sperm tracks with a straightness value less than 60% were considered non-progressive (Yogesh *et al.* 2018).

Sperm concentration: The sperm concentration was determined by using improved Neubauer's chamber as per standard protocol.

Sperm viability: Sperm viability was assessed as per Bloom (1950) and Hancock (1951) method. Dead spermatozoa could be differentiated by their ability to be stained by Eosin dye. One small drop of semen sample (kept at 37°C) was mixed with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide kept on a thermostatically warm stage (37°C). This mixture was kept for 2 min. A smear was then made from the mixture on a clean and grease free glass slide. It was dried in air and examined under the bright field 100× oil immersion objective of phase contrast microscope. Around 500 sperm were assessed in different five fields of a slide.

Acrosome integrity: Giemsa stain was used to assess the acrosomal integrity of stallion spermatozoa as per Watson (1975). Diluted semen drop was kept on clean grease free slide and thin smear was prepared. After air drying the smear slide was fixed in methanol for 15 min and then after washing, the fixed slide was kept in working solution of Giemsa for 90 min. Excess stain was removed by gentle stream of luke warm water. It was dried in air and examined under the bright field 100× oil immersion objective of phase contrast microscope. Around 500 sperm were assessed in different five fields of a slide.

Plasma membrane functional integrity: The plasma membrane integrity was determined through Hypo-osmotic swelling test (HOST) which is based on swelling ability of functioning sperms after being exposed to hypo-osmotic solution (Jayendran *et al.* 1984). Hypo-osmotic solution of 150 mOsmol/L was prepared (Sodium citrate 7.35 g, Fructose 13.51 g and double distilled water up to 1000 ml). Sperm tail curling was recorded as an effect of swelling due to influx of water. A total of 500 spermatozoa were counted in different fields at 40× magnification under phase contrast microscope.

DNA integrity: For detecting the DNA integrity of spermatozoa, the method described by Prashant *et al.* (2019) was adopted. Two hundred sperms per sample were counted under the epifluorescence microscope (480/550 nm). Sperm heads with intact chromatin showed green fluorescence and those with denatured (non-intact DNA) chromatin had

orange-red or yellow fluorescence. The percentage (%) of spermatozoa with single-stranded DNA was calculated from the ratio of spermatozoa with red, orange, or yellow fluorescence to all spermatozoa counted per sample.

Statistical analysis: Data pertaining to fresh, equilibration and post thaw stages of cryopreservation was analysed by one-way ANOVA using Tukey's test for normality for all. The factorial model included the effect of reduced ascorbic acid and glutathione as independent variables and percent post thawed progressive motility, live sperm count, acrosome intact sperm, hypo-osmotic swelling positive sperm and DNA integrity as dependent variables. Data were subjected to ANOVA, using the Post hoc procedure from Statistical software package, version 20 (SPSS 20, SPSS Inc., Chicago, IL, US). For fresh, unextended semen the Paired t-test model was used in order to see the variability between stallions as well as between the two groups. All results are expressed as the mean $\pm$ standard error (SE), and were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Assessment of fresh semen is an integral part and is a prerequisite for semen cryopreservation. Fresh semen samples with 60% or above progressive sperm motility were considered appropriate for cryopreservation of semen. The seminal characteristics (Mean $\pm$ SE) as observed in present study are presented in Table 1.

The fresh seminal parameters observed in the present study are well in accordance with the previous observations recorded by various workers (Pal *et al.* 2009, Yogesh *et al.* 2018) and there is no significant difference observed in various seminal parameters except in sperm concentration. Variation in the semen volume could be due to difference in individual semen production (Pickett *et al.* 1976, Pickett and Shiner 1994) and the teasing time (Ionata *et al.* 1991). In the present study, progressive motility observed in freshly ejaculated semen of Marwari stallions using CASA ranged from 75.28 ± 2.00 to $77.50 \pm 2.50\%$ with an overall mean of $76.57 \pm 1.25\%$. The results were consistent with the previous observations made by Ravi *et al.* (2013) who found

Table. 1. Seminal parameters in fresh semen of Marwari horses (mean $\pm$ SE) (n=24)

Seminal parameter	Mean $\pm$ SE
Total ejaculate volume (ml)	44.75 $\pm$ 2.86
Gel volume (ml)	9.79 $\pm$ 1.51
Gel free volume (ml)	34.95 $\pm$ 2.65
pH	7.64 $\pm$ 0.05
Progressive sperm motility (%)	76.57 $\pm$ 1.25
Sperm concentration* ($\times 10^6$ /ml)	215.37 $\pm$ 5.75 ^A
Sperm viability (%)	81.10 $\pm$ 1.70
Sperm plasma membrane integrity (HOST) (%)	57.13 $\pm$ 0.84
Acrosomal integrity (%)	86.07 $\pm$ 2.00
DNA integrity (%)	93.85 $\pm$ 0.33

^ASignificant difference between the stallions ($P < 0.05$).

Table. 2. Seminal parameters of Marwari stallion semen during pre-freeze and post-thawed semen after supplementation with antioxidants

Stage of Cryopreservation Group	Pre-free				Post-thaw					
	Progressive motility	Live dead ratio	HOST	Acrosome integrity	DNA integrity	Progressive motility	Live dead ratio	HOST	Acrosome integrity	DNA integrity
Ctrl	73.15±1.61 ^A	67.71±0.35 ^A	37.03±1.35 ^A	76.43±1.87 ^A	91.08±0.02 ^A	38.18±3.82	42.96±1.64 ^A	25.08±1.12 ^A	74.72±1.56	83.98±0.07 ^A
AA	78.94±1.42 ^B	74.91±0.62 ^B	42.58±1.62 ^B	81.27±1.00 ^B	92.56±0.08 ^B	42.12±3.03	55.00±2.05 ^B	36.18±1.79 ^B	77.23±1.43	84.76±0.05 ^B
GSH	78.78±2.36 ^B	78.12±0.85 ^C	48.43±1.18 ^C	82.91±1.20 ^{BC}	93.02±0.06 ^C	48.84±2.47	60.76±1.65 ^C	42.17±1.77 ^C	76.13±1.71	85.38±0.05 ^C
AA+GSH	77.16±2.71 ^B	80.89±0.76 ^C	50.22±1.53 ^C	85.39±1.09 ^C	93.75±0.09 ^D	46.96±2.74	65.32±1.06 ^C	44.35±1.85 ^C	78.17±1.62	86.33±0.06 ^D

Values bearing different superscripts within column differ significantly ($P < 0.01$).

progressive sperm motility in gel free stallion semen $77.00 \pm 1.51\%$. Similar observations were made by Pal *et al.* (2009) for Marwari breed who found an average motility of 73.33%. The range of sperm concentration in Marwari stallion recorded in the present study was 191.16 ± 4.11 to 237.66 ± 11.82 million/ml. Ravi *et al.* (2013) in their study found the values in a wide range from 115 to 275 million/ml, while Pickett *et al.* (1988) observed the values 100 to 200 million/ml in stallions' semen. Cueva *et al.* (1997) reported the average concentration of stallion semen 240.00 ± 0.10 million/ml. Soni (2016) found slightly higher sperm concentration in Indian horses with an overall mean of 260.55 ± 5.86 million/ml. Various factors affect the sperm concentration, viz. sperm concentration decreases with increasing number of mounts other than this, individual stallion, testicular circumference, teasing time before mount, season may influence the sperm concentration.

A decrease in almost all qualitative seminal parameters was observed because during freezing, sperm is exposed to severe osmotic, thermal, and oxidative stress, which damaged the plasma membrane and other spermatid structures such as acrosome and impaired the chromatin structure. A significant decrease ($P < 0.05$) in the progressive motility and percentage of sperm with an intact plasmatic membrane in post-thaw semen was observed as compared with fresh, pre-freeze, and frozen-thawed stallion semen (Table 2). This infers the direct effect of cryopreservation on diminishing the progressive motility and viability of spermatozoa (Fig. 1). Cryopreservation had a significant adverse effect on the other qualitative seminal parameters such as sperm plasma membrane functionality and acrosome membranes. There was a significant ($P < 0.05$) reduction in the number of spermatozoa with functional plasma membrane at fresh and post-thaw stage, and this difference seems to be narrowed at 2 h of incubation during the pre-freeze stage. A significant decline ($P < 0.05$) in the number of spermatozoa with intact acrosome was recorded at fresh, pre-freeze, and post-thaw stages of jack spermatozoa (Fig. 1), and this difference was found to be non-significant from the pre-freeze stage to the post-thaw stage. Similar kind of observations was made in case of the DNA intactness of the stallion spermatozoa.

The effect of supplementation of semen extender with different antioxidants, i.e. AA and GSH alone or in combination on sperm progressive motility, viability, and integrity of various sperm membranes of cooled and frozen-thawed stallion spermatozoa is presented in Table 2. A significant increase in the motility and the number of live spermatozoa was recorded in the semen samples supplemented with AA, GSH, or AA + GSH ($P < 0.05$). Among the antioxidants, combination of both the antioxidants (AA + GSH) groups showed marked increase in the motility and viability of stallion semen compared with the AA or GSH alone, and at the same time GSH over the AA proved to be a better antioxidant in increasing the motility and protecting the membranes (Table 2). The present study results are in accordance to that of previous

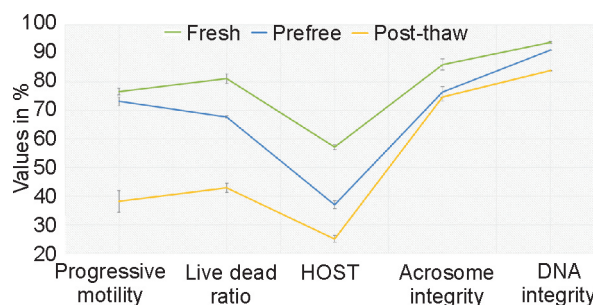


Fig. 1. Seminal parameters of Marwari stallion spermatozoa at fresh, pre-freeze and post-thaw stages.

studies carried out on GSH supplementation to the horse semen (Khelifaoui *et al.* 2005, Phetudomsinsuk *et al.* 2009), jack semen (Prashant *et al.* 2019) and boar semen (Estrada *et al.* 2015). However, Baumber *et al.* (2005) did not observe an improvement in total and progressive motility after the addition of 10 mM GSH. A protective effect of AA for membrane integrity was observed, but a deleterious effect on progressive motility was reported.

In the present study, non-significant difference ($P > 0.05$) was found among groups in post-thaw semen of Marwari stallions for acrosome integrity. Addition of antioxidants (AA and GSH) alone or in combination has significantly provided the cryoprotective effect to the spermatozoa during the cryopreservation process, which is evident from the data presented in Table 2. Membrane functional integrity yielded higher values in antioxidant groups than the control group ($P < 0.05$). Supplementation of the freezing media with amino acids (AA + GSH) had showed significant ($P < 0.05$) increase in the stability and maintaining the DNA integrity when compared with the control group semen samples both at incubation and after thawing process. The percentages of sperm with intact DNA did not significantly differ among the control, AA and GSH groups at both pre-freeze and post-thaw stages

Among the two antioxidants, GSH over AA proves to be a better antioxidant, as use of AA might be controversial, based on the previous studies, which stated that it may also act as a pro-oxidant in the presence of transition metals and it may make free radicals highly reactive and more destructive, thus generating more free radicals. There is ambiguity in the literature for the protective effect of antioxidants added to semen extenders. Some studies reported their positive effects, whereas others documented no benefit of addition of antioxidants. In contrast, Aurich *et al.* (1997) observed that AA had a protective effect on sperm membrane integrity of diluted stallion sperm. Likewise, the addition of GSH to freezing extender improved post-thaw sperm quality in bulls (Gangwar *et al.* 2018). The discrepancy in the results within species may be due to variations in age, animal breed, diluent components, semen conservation procedures, doses, and combinations of antioxidants.

There is discrepancy in the literature for the protective effect of antioxidants addition to semen extenders. Some studies reported positive effects, while others documented

no benefit of addition of antioxidants (Ball *et al.* 2001, Baumber *et al.* 2005, Gadea *et al.* 2007, Maia and Bicudo, 2009, Silva *et al.* 2009, Oliveira *et al.* 2013). The disparity in the results within species may be due to variations in age, animal breed, diluent components, semen conservation procedures, doses and combinations of antioxidants.

The process of cooling and freezing of semen has shown significant deteriorative effect on the different seminal quality parameters. Addition of antioxidants to the seminal plasma or extenders can be used for freezing to improve sperm viability and consequently, fertilizing capacity, by reducing oxidative effects caused by ROS. From the current study, it is inferred that supplementation of antioxidants (ascorbic acid and Glutathione) to the freezing extender has not only reduced the oxidative stress provoked by cryopreservation process but also preserved and enhanced various seminal qualitative parameters. And a combination of antioxidants than the single antioxidant supplementation proved to be significantly efficient in preserving the sperm from the cryodamage effect and they could be used effectively for semen cryopreservation in stallions.

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