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Effect of cysteine supplementation in semen extender on freezability and fertility of Murrah buffalo semen

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ABSTRACT

The present study was designed to evaluate the effect of different concentrations of cysteine on freezability and *in vivo* fertility of buffalo bull spermatozoa. Twenty-four ejaculates from four healthy breeding Murrah buffalo bulls were collected using artificial vagina. Qualified semen ejaculates (1-2 ml volume; >70% initial progressive motility; ≥4 mass activity; 1.0 billion/ml concentration) were diluted with Tris-citric acid extender containing 0.0 mM (control), 2.5 mM, 5.0 mM, 7.5 mM and 10.0 mM cysteine at 37°C and cryopreserved following standard protocol. The post-thaw semen was evaluated for CASA-based sperm motion traits, viability, plasma membrane integrity, acrosome integrity and oxidative stress. The sperm total and progressive motility, viability and plasma membrane integrity were higher in extender containing 2.5 mM/ml of cysteine compared to other concentrations. Acrosome integrity exhibited no difference in all groups with cysteine compared to control. Regarding sperm kinematics, addition of cysteine (2.5 mM/ml and 5.0 mM/ml) to extender significantly improved VCL and VAP as compared to other groups. The oxidative stress (MDA production) was significantly lower in extender supplemented with cysteine (5.0 mM/ml and 2.5 mM/ml) than in other groups. A total of 40 artificial inseminations were performed with the best evolved cysteine-supplemented extender (2.5 mM) based on the evaluation of post-thaw sperm attributes and control (20 each). *In vivo* fertility rates of buffalo semen were recorded non-significantly higher with extender supplemented with cysteine (2.5 mM; 55%) compared to control (0.0 mM; 45%). In conclusion, supplementing 2.5 mM cysteine in extender improved the post-thaw quality of Murrah buffalo bull semen.

Keywords: Buffalo, Cryopreservation, Cysteine, Fertility, Oxidative stress, Sperm characteristics

Semen cryopreservation is a well-developed technique that prolongs the life of spermatozoa. Successful freezability along with acceptable fertility of semen is essential to obtain maximum benefits from artificial insemination. However, owing to lower sperm freezability its use has been reported on a limited scale in buffalo. Previous reports (Kumar *et al*. 2014) have shown that in India, only 15% breedable buffaloes are bred through artificial insemination due to poor semen freezability which is primarily due to damage of plasma membrane integrity during freeze-thaw procedures, resulting in economic losses to AI industry. Moreover, sperm membrane is more predisposed to structural damage during cryopreservation due to lipid peroxidation through generation of free radicals (Kadirvel *et al*. 2009). Andrabi (2009) stated that sperm cryoinjuries have been positively linked to fertility in buffaloes. The number and quality of spermatozoa is determined pre-production through optimization of semen quality by dietary supplementation of nutraceuticals and post-production by *in vitro* treatment of semen with additives (Memon *et al*. 2012). Previously, use of various additives during cryopreservation has shown variable results on post-thaw semen quality across a variety of species (Nasiri *et al*. 2012). Detailed analysis revealed a need to identify suitable additive for minimizing the loss due to cryodamage in order to enhance the freezability of buffalo bull spermatozoa.

Amongst various additives, amino acids have been considered very potent due to their beneficial effects on semen quality (Bucak *et al*. 2008). Amino acids are antioxidant compounds that play a significant role in oocyte fertilization and protect the sperm cells from oxidative reactions during cryopreservation (Esmaeili *et al*. 2015). Cysteine is a sulfur-containing amino acid, naturally found in seminal plasma and sperm nucleic acid that maintains plasma membrane integrity and causes oxidation of hydrogen peroxide through direct chemical interactions with free radicals and protects DNA (Topraggaleh *et al*. 2014). Nevertheless, the concentration of cysteine present naturally in the semen gets depleted and consequently decreases from 41 mM to 29 mM during cryopreservation (Ugur *et al.* 2020). Previous studies have shown that addition of cysteine in extender improved the quality of semen in Holstein bulls (Bilodeau *et al*. 2001) and Alpine bucks (Anghel *et al*. 2010). However, studies regarding

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buffalo bull semen are limited and sparse. Keeping in view the above facts, the present work was undertaken to assess the effect of cysteine supplementation in extender on freezability and *in vivo* fertility of buffalo bull spermatozoa.

MATERIALS AND METHODS

Semen collection, evaluation, processing and cryopreservation: The *in vitro* studies on cryopreserved buffalo bull semen were approved by the Institutional Animal Ethics Committee (IAEC) of the University (61/04). For conducting fertility trials (artificial insemination and ultrasonography), the study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; F. No. 25/1/2018-CPCSEA). In the present study, twenty-four semen ejaculates were collected from four breeding buffalo bulls (six ejaculates per bull; aged between 4-6 years and having body weight between 700-800 kg), routinely used in breeding programme and having proven fertility record at different intervals. The bulls were maintained on a regular feeding schedule as per NRC recommendations including green fodder (40-45 kg/bull/day), wheat straw (2-3 kg/bull/day), concentrate (4 kg/bull/day) and mineral mixture in the concentrate (2%). Using artificial vagina, semen from each bull was collected twice in a week in the morning hours during the months of September through November. To ensure that the sperm reserve from each bull was empty, two semen collections were conducted prior to the start of the experiment. Each ejaculate was divided into five equal fractions, diluted to a final concentration of 80×10^6 sperm/ml through pre-calibrated and automated Accucell Photometer (IMV Technologies, L'Aigle, France) using Tris-egg yolk semen extender (100 ml containing 20 ml Egg yolk, 7 ml glycerol, 73 ml Tris buffer and 5 mg gentamycin) supplemented with various doses of cysteine (catalog no. 168149, Sigma-Aldrich, St. Louis, USA) such as Control Group (0.0), Group I (2.5), II (5.0), III (7.5) and IV (10.0) mM/ml, respectively to evaluate the most effective cysteine concentration for its cryoprotective effect using 7% glycerol as cryoprotectant. Only ejaculates with initial progressive motility 70%, mass activity ≥4 and concentration 1.0 billion/ml subjectively assessed under a phase contrast microscope equipped with a stage warmer at 37°C and under 40× magnification were used in the study. The diluted semen in each ejaculate was allowed to equilibrate at 4°C for 3-4 h in a cold handling cabinet. Following equilibration, the semen was loaded into 0.25 ml plastic straws automatically through a filling and sealing machine (IMV Technologies L'Aigle, France) and cryopreserved. All the straws were exposed to liquid nitrogen (LN_2) vapors for about eight minutes at 4 cm height and then plunged in LN_2 for storage pending analysis. Following cryopreservation, the examination of frozen-thawed semen was performed within a week.

Evaluation of post-thaw sperm functional assays: The post-thaw semen of each ejaculate was examined for CASA-based sperm motility and kinetics, viability, plasma membrane integrity, acrosome integrity and lipid peroxidation to determine the most effective dose of cysteine.

Assessment of sperm motility and kinematics: Sperm motion traits were evaluated using a previously calibrated computer-assisted semen analysis (CASA; Androvision, Minitube, Germany). Exactly 10 µL of frozen-thawed semen was placed on a pre-warmed disposable glass slide to analyze the CASA-based sperm motion traits. The average of five optical fields of the glass slide was used to count spermatozoa in each semen sample.

Sperm viability: The live sperm count was determined by Hoechst 33342 and PI fluorescence staining method as described by Pintado *et al.* (2000). The frozen semen straw was thawed at 37°C for 30 s. Exactly 1.5 µL of Hoechst 33342 and PI stains were mixed to 50 µL of frozen-thawed semen in an eppendorf tube (2 ml) at 38°C. The semen stain mixture was incubated in a dark chamber at 38°C for 15 minutes to protect from light. Then, 10 µl of the pre-warmed sample was placed on a microscopic slide and covered with a cover slip. The stained sperm cells were identified under microscope at 20× magnification of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The PI stain permeates only damaged sperm membranes which appear red. The Hoechst 33342 dye binds specifically to A-T regions of DNA and emit blue colour. Accordingly, the spermatozoa which emitted red fluorescence were considered dead and those which reflected blue fluorescence were live. Thereafter, module "viability" of Androvision was selected to record the percentage of viable sperm objectively.

Sperm plasma membrane integrity: Plasma membrane integrity was examined using hypo-osmotic solution (150 mOsm/L of hypo-osmotic solution containing 9.9 g D-fructose, 4.9 g Trisodium citrate, DDW up to 1000 ml; Jeyendran *et al.* 1984). The test was performed by mixing 100 µl of semen to 1.0 ml of HOS solution. Simultaneously, 1.0 ml of phosphate buffer saline (control) was added to 100 µl semen. The semen aliquots were then incubated for 1 h at 37°C. Exactly 10 µl incubated semen from HOS and control was placed on separate glass slides and cover slips were placed over them. The semen in both glass slides was observed at 40× magnification under phase contract microscope. About 200 curled and non-curled spermatozoa were counted in both HOS and control aliquots and expressed in percentage. The number of curled spermatozoa in control were subtracted from that in HOS in order to obtain HOS-reactive spermatozoa.

Acrosome integrity: Acrosome integrity was determined by Hoechst 33342 and FITC-PNA fluorescence staining method as described earlier (Fischer *et al.* 2010). The frozen semen straw was thawed at 37°C for 30 s. Exactly 27 µl of Hoechst 33342 and FITC-PNA stains were mixed to 50 µl of frozen-thawed semen in an Eppendorf tube (2 ml) at 38°C. The semen stain mixture was incubated in a dark chamber at 38°C for 20 min to protect from light. Then, 10 µl of pre-warmed sample was placed on a microscopic slide and covered with a cover slip. The stained sperm cells

were identified under microscope at 20× magnification of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The FITC-PNA stain permeates only damaged acrosomes which appear green. The Hoechst 33342 dye binds specifically to A-T regions of DNA and emits blue color. Accordingly, the spermatozoa which emitted green fluorescence were considered as damaged acrosomes and those which reflected blue fluorescence were considered as intact acrosomes. Thereafter, module "viability" of Androvision was selected to record the percentage of intact acrosomes objectively.

Lipid peroxidation (LPO): LPO in post-thaw semen was evaluated by determining the Malondialdehyde (MDA) levels using TBA-TCA (thiobarbituric acid-trichloroacetic acid). Briefly, the sperm pellet was suspended in PBS (*p*H 7.2) to obtain a concentration of 20 million/ml. Exactly 1.0 ml of sperm suspension was added into 2 ml of TBA-TCA reagent and heated in the boiling water for 1 h. The colour of the solution was turned from transparent to yellowish and samples were allowed to cool at room temperature. Centrifugation of the samples was done at 3000 rpm for 10 min at room temperature. The supernatant was separated and absorbance was read in bio-spectrophotometer (Eppendorf, Hamburg, Germany) at 535 nm against blank solution. The calculation of MDA value (µmol/20 million sperm/ml) was done as per the formula given below:

$$\text{MDA (µmole/20 million sperm/ml)} = \frac{\text{O.D. ×106× Total volume of reaction mixture (3 ml)}}{\text{1.56 ×105× Total sample volume (1 ml)}}$$

$$\text{MDA (µmole/20 million sperm/ml)} = \frac{\text{O.D} \times 30}{1.56}$$

Where, 1.56 is specific absorbance coefficient.

In vivo fertility rate: She buffaloes (40) maintained under standard feeding and management systems at organized farms were enrolled for fertility trail. All the buffaloes were pluriparous (2nd to 5th parity), apparently healthy and free from physical and/or reproductive problems. Two experimental extenders having cysteine 0.0 mM/ml (control) and 2.5 mM/ml (most optimal concentration based on the assessment of sperm characteristics) were prepared (as described earlier) and used for artificial insemination (20 each). All the buffaloes were monitored until ovulation using a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency of ultrasound machine (Sonosite, Mturbo, France). The buffaloes were inseminated on observed estrus and following 12 h in the same cycle after confirmation with per-rectal examination and ultrasound scanning by same person to minimize the variations due to the insemination technique, time of insemination, semen handling and site of semen deposition, if any. The pregnancy diagnosis was performed on day 60 post-insemination using ultrasonography. The first service pregnancy rate (FSPR) was calculated according to the following method:

$$\text{FSPR (\%)} = \frac{\text{Number of buffaloes pregnant after first insemination}}{\text{Total number of first services}} \times 100$$

Statistical analysis: Statistical evaluations were carried out using the GraphPad Prism 8 Software, version 10.2, GraphPad Software Inc., California, USA. The proportionality data were estimated following arcsine transformation to normalize data. One way Analysis of Variance (ANOVA) with the Bonferroni test was used for comparisons of means among treatments. The data pertaining to *in vivo* fertility rate was evaluated using Chi-square test. A confidence level ($P<0.05$) was considered to be significant for all analyses. Results were expressed as mean±SEM.

RESULTS AND DISCUSSION

Effect of supplementation of cysteine in extender on CASA-based motility traits in post-thaw buffalo sperm: The comparative efficacy of different concentrations of cysteine supplementation on post-thaw sperm motility and velocity parameters are presented in Table 1. The present study revealed that the percentage of total motility was significantly higher ($P<0.05$) in semen supplemented with 2.5 and 5.0 mM/ml than in their other experimental concentrations and control counterparts (Table 1). The progressive motility in extender containing 2.5 mM/ml

Table 1. CASA-based sperm motion traits (Mean±SEM) in post-thaw buffalo semen supplemented with different concentrations of cysteine (n=24 ejaculates)

Parameter	Cysteine (mM/ml)				
	Control (0.0)	2.5	5.0	7.5	10.0
TM (%)	44.6±2.0^{a}	51.20±1.8^{b}	50.9±1.4^{b}	45.0±1.7^{a}	42.0±1.4^{a}
PM (%)	34.9±1.6^{a}	40.7±2.0^{b}	36.4±2.0^{ab}	34.2±1.8^{a}	32.0±1.4^{a}
VSL (µm/s)	80.1±2.4^{a}	84.3±2.5^{ab}	87.3±2.2^{bc}	89.3±1.8^{c}	92.3±3.3^{c}
VAP (µm/s)	91.4±3.2^{a}	107.5±3.5^{b}	108.4±3.7^{b}	95.8±2.6^{a}	93.5±2.8^{a}
VCL (µm/s)	131.5±3.5^{a}	162.7±2.9^{b}	154.5±3.3^{c}	140.3±2.1^{d}	143.9±2.7^{d}
ALH (µm)	7.0±0.5	6.5±1.0	7.8±0.6	6.9 ± 0.7	6.7±0.9
BCF (Hz)	38.7±2.8	36.4±3.7	37.2±1.9	39.7±2.4	37.8±2.1

Mean values with different superscripts within the row differ significantly ($P<0.05$). TM, Total motility; PM, Progressive motility; VSL, Velocity straight line; VAP, Velocity average path; VCL, Velocity curvilinear; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency.

cysteine was non-significantly higher (P>0.05) as compared to that supplemented with 5.0 mM/ml but significantly higher (P<0.05) than 7.5 mM/ml, 10.0 mM/ml and control. These findings are in consonance with the observations of Iqbal *et al*. (2016) in buffalo bulls who reported higher (P<0.05) sperm motility in semen extended with 2.5 mM/ml cysteine (67.24±3.74%) than in control (52.46±5.20%). Likewise, supplementation of 2.5 mM/ml cysteine in extender exhibited higher (P<0.05) sperm motility (58.3±2.9%) as compared to that with 0.5 mM/ml (41.7±3.0%) and control (41.7±2.9%) in Sahiwal bulls (Ansari 2011). In Yorkshire boars, Chanapiwat and Kaeoket (2021) also found higher (P<0.05) sperm motility (71.3±4.8%) in semen extended with 2.5 mM/ml cysteine in comparison to that with 5.0 mM/ml (46.9±14.9%), 10.0 mM/ml (45.0±19.2%) and control (51.3±4.4%). High sperm motility in semen supplemented with 2.5 mM/ml cysteine could be due to its specific composition. Previous studies of Bilodeau *et al*. (2001) have shown that cysteine plays a pivotal role in reinforcement of sperm plasma membrane, protection against cold shock and decline in lipid peroxidation (LPO). During cryopreservation there is damage to axoneme and mitochondria in spermatozoa. Inclusion of cysteine in extender regulates cryoprotective effects on the integrity of axosome and associated dense fibers in the middle piece of spermatozoa that contain mitochondria and generate energy from intracellular stores of ATP which might contribute towards better buffering capacity and subsequently higher sperm motility (Ansari 2011). However, at lower or higher concentrations than 2.5 mM/ml, cysteine may act as an oxidation stimulator rather than an antioxidant (Sarıözkan *et al*. 2009). Moreover, at altered concentrations of cysteine, generation of excessive reactive oxygen species (ROS) by spermatozoa also has been linked to reduced sperm motility during cryopreservation (Beheshti *et al*. 2011).

CASA-based sperm velocity parameters are depicted Table 1. Among various sperm kinematic traits, supplementation of 2.5 mM/ml cysteine significantly (P<0.05) improved velocity curvilinear (VCL) when compared to other experimental concentrations and control. Velocity average path (VAP) was significantly higher (P<0.05) in extender supplemented with 5.0 mM/ml and 2.5 mM/ml cysteine as compared to their counterparts although no difference (P>0.05) was ascertained between the former concentrations. While a dose-dependent response was noticed in respect of velocity straight line (VSL) in the tested concentrations and control, no change was observed for amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). A similar dose-response in VSL following addition of cysteine analogue in extender was noticed by Tuncer *et al*. (2010) in the cryopreserved sperm of Holstein bulls. They also recorded higher (P<0.05) values of VAP at lower concentrations as compared to higher ones and control. Excessive generation of ROS increased the proportion of impaired cells and exhibited adverse effects on sperm velocity parameters was observed in concentrations more than 2.5 mM/ml cysteine and control (Metwaly *et al*. 2016).

Effect of supplementation of cysteine in extender on sperm function assays in post-thaw buffalo sperm: In order to achieve optimal post-thaw sperm fertility, the plasma membrane should remain intact and viable. Amongst different concentrations, the proportion of viable spermatozoa was significantly higher (P<0.05) in the Tris-egg yolk extender supplemented with 2.5 mM/ml cysteine than in their counterparts (Table 2, Fig. 1). The results of the current study are in line to an earlier report by Ansari (2011) who reported increased (P<0.05) cell membrane resistance to cryo-damages following supplementation of cysteine at 2.5 mM/ml (79.3±4.2%) than at 1.0 mM/ml (73.3±1.5%), 0.5 mM/ml (62.7±1.5%) and control (61.7±2.5%) in Sahiwal bulls. Similarly, addition of semen extender with 2.5 mM/ml cysteine as compared to 0.5 mM/ml and control significantly (P<0.05) improved sperm viability (51.5±1.04% vs 39.6±1.25% vs 36.0±0.93%) in Baladie goats (Metwaly *et al*. 2016). In another report, Güngör *et al*. (2017) also noticed that semen supplemented with 2.5 mM/ml cysteine than that with 4.0 mM/ml and control resulted in significantly (P<0.05) higher percentage of viable sperm (57.25±3.57 vs 49.08±4.50 vs 46.98±4.08) in Merino rams. Addition of cysteine in the extender might have replaced and/or replenished antecedently lost amino acid concentrations during freeze-thaw stages, thereby reduced the formation of ice crystal, increased the tolerance limits of osmosis and subsequently improved sperm cryostability (Funahashi and Sano 2005). Moreover, covalent bonds formed between amino acid side chains in plasma membrane by ROS might be disrupted by cysteine and maintain membrane fluidity (Güngör *et al*. 2017).

Table 2. Sperm function assays and oxidative stress (Mean±SEM) in post-thaw buffalo semen supplemented with different concentrations of cysteine (n=24 ejaculates)

Parameter	Cysteine (mM/ml)				
	Control (0.0)	2.5	5.0	7.5	10.0
Sperm function assays					
Viability (%)	57.2±1.7^{a}	66.4±1.9^{b}	62.5±2.2^{b}	56.3±1.7^{a}	59.6±2.7^{ab}
PMI (%)	42.5±1.2^{a}	48.5±1.3^{b}	42.6±1.0^{a}	43.2±1.2^{a}	44.0±1.7^{a}
Acrosome integrity (%)	59.3±2.4	57.1±1.6	60.5±2.8	58.4±2.0	55.7±2.7
Oxidative stress					
Malondialdehyde (µmole/20 million sperm/ml)	2.92±0.45^{a}	1.71±0.33^{b}	1.65±0.29^{b}	2.04±0.31^{b}	2.43±0.40^{ab}

Mean values with different superscripts within the row differ significantly (P<0.05).

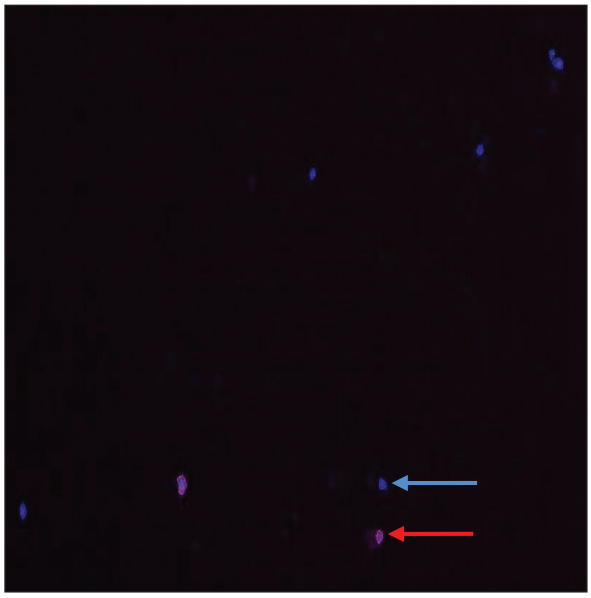

Fig. 1. Viability in post-thaw spermatozoa of buffalo bulls (20×). Sperm which emit blue fluorescence are considered live, designated by blue arrow. Sperm which emit red fluorescence are considered dead, designated by red arrow.

Seminal plasma of bulls contains natural amino acids. Eventually, upon dilution of fresh ejaculate, concentration of these amino acids usually declines. Addition of additives containing amino acids in appropriate levels to freezing extender helps in preserving sperm composition following cryopreservation (O'Flaherty *et al*. 1997).

The primary prerequisite for ideal sperm function and metabolism relies on the intactness of plasma membrane. Any damage to the structural integrity of plasma membrane of spermatozoa results in loss of homeostasis and cellular death. The mean sperm plasma membrane integrity in post-thaw semen is shown in Table 2. The current study revealed that plasma membrane integrity was significantly higher ($P<0.05$) in semen supplemented with 2.5 mM/ml cysteine as compared to other experimental concentrations and control. Concentrations higher or lower than 2.5 mM/ml resulted in lower plasma membrane integrity after freeze-thawing. The findings of our study are in accordance with the observations of Iqbal *et al*. (2016) in buffalo bulls who recorded higher plasma membrane integrity ($P<0.05$) in Tris citric egg yolk extender containing 2.5 mM/ml (38.50±0.54%) as compared to control (27.45±0.74%). In bulls, Sarıözkan *et al*. (2009) also depicted ($P<0.05$) higher plasma membrane integrity following supplementation of 2.5 mM/ml L-cysteine (57.8±1.2%) as compared to control group (49.6±1.1%). Similarly, addition of cysteine at 2.5 mM/ml than at 5.0 mM/ml, 0.5 mM/ml and control revealed higher ($P<0.05$) proportion of intact plasma membrane (53.6±1.18 vs 43.1±1.51 vs 37.1±1.05 vs 37.8±1.30, respectively) in goats (Metwaly *et al*. 2016). Sperm survival is largely dependent on the intact plasma membrane and has been linked with improved fertility *in vivo* (Perez-Llano *et al*. 2001). Sperm membrane lacks antioxidant-rich amino acid component, making them extremely vulnerable to LPO (Saleh and Agarwal 2002). As reported earlier, cysteine strengthens the plasma membrane of spermatozoa and plays a key role in reducing LPO (Asadpour *et al*. 2012). Moreover, conversion of cysteine to taurine and acetyl–taurine play an important role in osmoregulation of sperm membrane through reduced superoxide anion and decreased LPO thereby enhanced protection of plasma membrane against ROS (Sarıözkan *et al*. 2009). Hence, in the current study, higher proportion of sperm with intact plasma membrane in extender supplemented with 2.5 mM/ml cysteine than in their counterparts could have resulted from lesser membrane damage owing to decreased production of free radicals and increased antioxidant enzyme activity (Iqbal *et al*. 2016).

The mean sperm acrosomal integrity in post-thaw semen is shown in Table 2. In the present study, the percentage of intact acrosomes did not seem to be affected following addition of cysteine since no difference ($P>0.05$) were noted across all treatments (Fig. 2). These findings are in agreement with the observations of Baheshti *et al*. (2011) who depicted that supplementation of cysteine in extender did not affect ($P>0.05$) acrosome integrity in buffalo semen (68.46±0.21% at 5.0 mM/ml, 67.85±0.52% at 7.5 mM/ml, 67.98±0.11% at 2.5 mM/ml and 68.38±0.23% in control). No difference ($P>0.05$) in the proportion of intact acrosomes was seen among different cysteine complemented groups (57.55±2.05% at 2.5 mM/ml, 56.2±3.78% at 5.0 mM/ml, 56.40±03.70% at 8.0 mM/ml) and control group (54.56±3.48%) at post thaw stage in buffalo semen (Wadood *et al*. 2015). Similarly in goats, supplementation of cysteine at different concentrations had no effect ($P>0.05$) on acrosome integrity in post-thaw stage (59.2±0.4%:5.0 mM/ml; 58.1±0.43%:10.0 mM/ml;

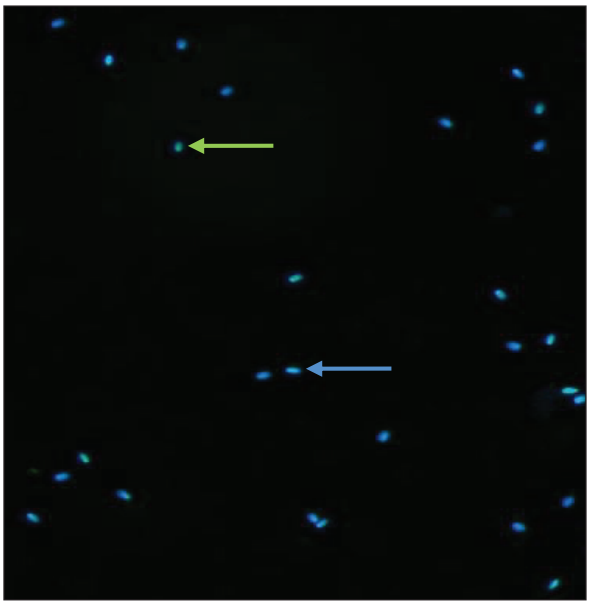

Fig. 2. Acrosome integrity in post-thaw spermatozoa of buffalo bulls (20×). Sperm which emit blue fluorescence indicate intact acrosomes designated by blue arrow and sperm which emit green fluorescence indicate damaged acrosomes designated by green arrow.

Table 3. Fertility results of post-thaw buffalo semen supplemented with cysteine

Extender	No. of Buffaloes inseminated	No. of buffaloes conceived	FSPR (%)	Chi square	P- value
Control (0.0 mM/ml)	20	9	45.0	0.400	0.527^{NS}
Cysteine (2.5 mM/ml)	20	11	55.0		

NS, Non-significant.

58.1±0.52%:20.0 mM/ml and 57.2±0.41% in control group; Memon *et al*. 2012). For zona penetration and successful fertilization, functional as well as morphological intactness of acrosome is critical (Yu *et al*. 2006). During cryopreservation generation of ROS molecules can induce damage to acrosome (De Lamirande *et al*. 1997). Cysteine has a scavenging role on ROS products through enhanced antioxidant activity and plays an important role in protection of acrosomes from impairment (Bucak *et al*. 2008). However, in the current study, supplementation of cysteine to semen extender revealed no change in the percentage of sperm with intact acrosomes among all treatments as well as in control. Khalifa *et al*. (2008) reported that cysteine being a water-soluble antioxidant does not bind properly to acrosome, thereby, reflecting the null effect on maintaining the acrosomal integrity/intactness of the spermatozoa.

Effect of supplementation of cysteine in extender on oxidative stress in post-thaw buffalo sperm: The end product of LPO is evaluated by the amount of malondialdehyde (MDA) produced. Changes in mean MDA (µmole/20 million sperm/ml) concentrations in semen supplemented with 2.5, 5.0, 7.5 and 10.0 mM/ml and control (0.0 mM/ml) have been given in Table 2. Addition of different concentrations of cysteine to the freezing media revealed that the levels of MDA produced at concentrations 5.0 mM/ml (1.65±0.29 µmole/20 million sperm/ml) and 2.5 mM/ml (1.71±0.33 µmole/20 million sperm/ml) were non-significantly lower (P>0.05) than at 7.5 mM/ml (2.04±0.31 µmole/20 million sperm/ml) and significantly lower (P<0.05) when compared to that at 10.0 mM/ml (2.43±0.45 µmole/20 million sperm/ml) and control (2.92±0.40 µmole/20 million sperm/ml). Nonetheless, compared to control the MDA production was lower in all cysteine supplemented groups. Literature on the impact of cysteine supplementation in extenders on MDA levels in buffalo semen is not available. However, comparative studies in other species (buck) also showed that supplementation of cysteine had lower (P<0.001) MDA concentrations (66.49±4.12 nmol/ml at 2.5 mM/ml, 67.70±4.26 nmol/ml at 5.0 mM/ml and 53.91±1.57 nmol/ml at 10.0 mM/ml) compared to control (83.75±5.38 nmol/ml; Altyeb *et al*. 2022). Although non-significant, Adekunle *et al*. (2022) also noted lower (P>0.05) MDA levels in cysteine supplemented groups (0.22±0.03 nmol/ml at 2.0 mM/ml, 0.20±0.01 nmol/ml at 4.0 mM/ml, 0.11±0.03 nmol/ml at 6.0 mM/ml and 0.15±0.03 nmol/ml at 8.0 mM/ml) than in control group (0.58±0.01 nmol/ml) in West African goat bucks. During storage, excessive production of ROS by leukocytes and/or spermatozoa is detrimental to sperm function (Agarwal and Said 2003). Cysteine is a potent antioxidant that plays an important role in maintaining sperm function through inhibition of LPO (Johnson *et al*. 2000). In the current study, cysteine @ 2.5 mM/ml might have lowered MDA production leading to reduced oxidative stress to sperm cells in extender.

Effect of supplementation of cysteine in extender on in vivo fertility of post-thaw buffalo sperm: The data on first service pregnancy rate (FSPR) of post-thaw buffalo semen containing 2.5 mM/ml cysteine and control has been presented in Table 3. In the present study, the FSPR was non-significantly higher using semen supplemented with 2.5 mM/ml cysteine (55%; 11/20) compared to control (45%; 9/20). Although, the number of animals inseminated in each group was relatively small, supplementation of cysteine tended to increase the FSPR (an increase of 10.0 percent) in comparison to their control counterparts. Determination of fertility rate gives a more legitimate picture of the post-thaw semen quality (Singh *et al.* 2017). Earlier studies (Iqbal *et al*. 2016) in buffalo bulls have also depicted higher (P>0.05) fertility rates with cryopreserved semen containing 2.5 mM/ml cysteine as compared to control ones (59% vs 43%). In another study in buffalo semen, Ansari (2016) also recorded better conception rates following supplementation of tris-citric acid extender with 2.5 mM/ml cysteine (55%; 55/100) than in control (43%; 43/100). Similar studies (Sarıözkan *et al*. 2009) in bulls also stated higher (P>0.05) fertility rate following insemination with semen containing 2.5 mM/ml cysteine (74%) than in control (57%). During cryopreservation, there is impairment of plasma membrane, changes in fluidity and altered influx of calcium (Collin *et al*. 2000). Supplementation of cysteine might have counteracted peroxidation, stabilized membranes and decreased calcium uptake that consequently improved post-thaw sperm survival resulting in better fertility rates in the present study.

In conclusion, cysteine (@ 2.5 mM/ml) supplementation in extender improves the post-thaw sperm characteristics and may be adopted in routine semen cryopreservation protocols for better outcomes of cryopreserved semen.

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