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Impact of Cysteine as a Semen Additive on Mitigating the Decline in Sperm Quality due to Summer Stress in Riverine Buffaloes (*Bubalus bubalis*)

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ABSTRACT

The seasonal variations such as temperature, rain and humid environment greatly influences reproduction, and semen quality particularly in buffalo bulls, either positively or negatively. The heat stress coupled with freezing and thawing processes generates reactive oxygen species (ROS), which further reduce post-thaw spermatozoa quality. Application of cysteine as a semen additive have been reported to alleviate such damaging effect in canine, buck, bull, human, boar, and rooster. Therefore, objective of this study was to investigate ameliorating effect of cysteine on summer stress on buffalo spermatozoa. For the experiment, 24 ejaculates with individual progressive motility >70, volume >1mL and sperm concentration 1000 million/mL from four Murrah buffalo bulls were collected and divided into four groups as Control (Group-I), and Group-II, III, and IV (4, 8, and 12 mmol cysteine, respectively) containing 80 million spermatozoa per mL. Semen was filled (French mini straw), sealed and equilibrated at 40◦C and frozen into LN_2. The group containing L-cysteine at 4 mmol concentration had higher significant (p<0.05) difference in post thaw motility, viability, plasma membrane integrity and acrosome integrity, TAC, DNA integrity and lower significant (p<0.05) difference in spermatozoa abnormality, LPO and intracellular ROS. Moreover, cysteine concentration at 8 mmol has no beneficial effect compared to the Control, whereas Group-IV had greater adverse effect. This may be due to its toxicity at this concentration. It is evident from the study that the cysteine molecules at 4 mmol concentration produced more pronounced improvement in the freezability potential of buffalo bull spermatozoa.

Key words**:** Buffalo, Cysteine, Spermatozoa, Free radicals, ROS, Summer

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INTRODUCTION

India has 109.8 million buffalo (approximately 57.7% global buffalo population, DAHDF, Govt. of India, 2019-2020), contributing more than half of the total milk production producing 91.82 million tons of milk in the year 2019. Buffaloes, rightly called 'Black gold' serve as a source of meat and traction force, however, the climatic factors may contribute adversely or beneficially to the productivity and reproduction, including quality semen in buffalo. For the improvement of buffalo farming the breeding programme must retain superior quality fertile bulls to harvest quality semen in all seasons. It is to be noted that the semen characteristic influences the conception-rate and fertility-rate (Krishnan *et al*., 2017).

However, reports suggest that buffalo spermatozoa are primarily susceptible to the heat-stress (HS) during summer than other livestock animals (Ahirwar *et al.,* 2018). Production of quality spermatozoa is related to non-genetic and genetic factors, of which the non-genetic factors are majorly influenced by the climatic variation. Since buffaloes diminished quantity of sweat glands as compared to other livestock species it has less chance to alleviate the HS. Moreover, freezing-thawing processes coupled with HS generate Reactive oxygen species (ROS), which further reduce post-thaw semen quality (Bisla *et al* 2020). For, buffalo spermatozoa have low amount of cholesterol with comparatively greater head-size they are easily susceptible to HS (Kumar *et al*., 2021).

Recently, addition of amino acids like taurine and cysteine to the semen extender as an anti-oxidant was followed in boar (Funahashi and Sano, 2005), bull (Uysal *et al.,* 2007), ram (Bucak *et al*., 2007) and goat (Atessahin *et al.,* 2008) to improve the post-thaw semen-quality of spermatozoa. Though buffalo spermatozoa are highly susceptible to HS and ill-effects of ROS, report on incorporation of cysteine in extenders to counteract free-radicals was not available in the literature scanned. Cysteine and glutamine play a major role as antioxidants and prevent the HS by reducing lipid peroxidation in the spermatozoa (Tuncer *et al.,* 2010; Gaurav *et al*., 2012). Moreover, it is easily available and cost-effective product compared with other semen additives used for similar purposes. L-cysteine is naturally found amino acid having N-acetyl derivative (NAC, Aitken, 1993) and because of its capacity to reduce disulfide bonds, NAC is nearly nontoxic, commonly used to reduce mucus viscosity and elasticity (Ciftci *et al*., 2009). Thiol group in cysteine act as penetrating non-enzymatic antioxidants (Coyan *et al.,* 2011). Furthermore, the significant improvements of cysteine on spermatozoa chromatin-defense have been demonstrated for chilled boar spermatozoa preservation (Szcesniak-Fabianczyk *et al.,* 2003) and freezing bull spermatozoa (Tuncer *et al*., 2010). A clear effect of cysteine (Beheshti *et al*., 2011) and glutamine (El-Sheshtawy *et al*., 2008) on motility, intracellular ROS, mitochondrial membrane potential (MMP), and DNA damage of frozen–thawed buffalo spermatozoa have not been identified. Cysteine is thought to protect spermatozoa's acrosome integrity by harvesting ROS molecules through a glutathione-mediated process and increasing antioxidant activity in the semen-extender complex (Bucak *et al*., 2008). With this background, the objective of this study was to investigate ameliorative effect of cysteine as a semen additive to improve spermatozoa quality.

MATERIALS AND METHODS

The work was carried out at Germ Plasm Centre, Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). The institute is located at an altitude of 564 feet above the MSL at latitude of 28° N and longitude of 79° E. The subtropical climate of the place experiences both very hot and cold weather conditions with the relative humidity ranging between 15 to 85% in various months of the year.

Experimental animals and source of semen

Four healthy Murrah buffalo (*Bubalus bubalis*) bulls of 4-6 years age maintained at GPC were utilized for the current study. The recommended feeding and management practices were followed for bulls during the entire course of investigation. All the chemicals and stains used in the experiments were procured from Sigma-Aldrich (Germany) unless otherwise mentioned at the appropriate place.

Experimental bulls were housed in individual pen in an environmentally controlled building with exposure to natural day light. The semen samples were collected once between 8 AM to 9 AM during morning hours at 10 days interval, using artificial vagina following the standard procedure. Tubes containing the ejaculated semen were placed in a water bath at 37°C immediately after collection. Selection of the ejaculates was based on volume, sperm concentration and individual progressive motility. For the purpose of uniformity, throughout the experiment only those ejaculates that had a volume of >1.5-2.0 mL, sperm concentration >500 million/mL, mass activity of >3+ and individual progressive motility of >70% were selected for further evaluation and sampling. A total of 24 ejaculates from 4 Murrah buffalo bulls (6 x 4) were collected for the

present study. The sample was divided into four groups: Group I (Control), and treatment groups viz. Group II, Group III, and Group IV. In each of the above treatment group cysteine was added at the ratio of 4mmol, 8mmol and 12mmol, respectively in Group II, III, and IV, and extended with the TYG extender in such a way to achieve final concentration of 80 million/mL. Filling and sealing (IMV, France) of straws were done immediately. Following initial semen evaluation, samples were processed for cryopreservation using standard protocol.

Evaluation of semen at fresh stage

Collected samples were evaluated for mass motility, concentration (Accucell, IMV, France), Individual progressive motility, viability (Eosin-Nigrosine stain (Swanson and Bearden, 1951), Sperm morphological abnormalities (Rose Bengal (RB) stain), Acrosome integrity (Giemsa stain, Watson, 1975), and Hypo-osmotic swelling test (HOS, Jeyendran *et al.*, 1984) for plasma membrane integrity. Thereafter, ejaculates were regrouped based on THI values and the effect of THI on morphological abnormalities of fresh spermatozoa, and certain parameters at post thaw stage were assessed.

Evaluation of semen at post thaw stage

Straws were placed in water having temperature 37°C for 30s for thawing. After 30s, straws were taken out, wiped followed by cutting and pouring the content into glass sugar tube kept at 37°C in water bath. The semen sample was used immediately for evaluation of different semen quality parameters.

Semen from each group was evaluated for individual progressive motility, viability, biochemical integrity of plasma membrane (HOS), sperm abnormalities and acrosome integrity (Giemsa stain), expressed as percent employing methods as described earlier to determine effect of addition of cysteine as an additive at stage II (post-thaw stage). The nuclear integrity procedure involving acridine orange stain was used (Tjeda *et al.*, 1984), modified by Sadeghi *et al.* (2009). Total antioxidant status in seminal plasma was measured using ferric reducing/ antioxidant power (FRAP) assay (Benzie and Strain, 1996). The LPO of spermatozoa was assessed by determining the malondialdehyde (MDA) concentration in the sperm pellet via TBA-TCA (Thiobarbituric acid-trichloroacetic acid) method (Suleiman *et al.*, 1996). The intracellular ROS in spermatozoa was estimated using protocols of Carter *et al.* (1994) and Kasahara *et al.* (2002).

Statistical analysis

The generated data from the experiment was analysed by ANOVA using SPSS 20.0 statistical software and results were expressed as Mean ± standard error. Mean values between control and treatment groups and different stages were compared by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Buffalo spermatozoa are primarily susceptible to the heat stress, further exaggerated by the freezing and thawing processes affecting post thaw semen quality (Ahirwar *et al.*, 2018). This study, therefore, was undertaken to investigate the effect of cysteine as semen additive in countering detrimental effect of free radicals on post thaw semen quality.

Post thaw semen quality

Following addition of cysteine in the 3 treated groups and their comparative study with the control, processed semen samples were evaluated for post thaw motility, viability, integrities of plasma membrane, acrosome, and DNA, and spermatozoa morphological abnormalities.

The study results show greater mean value of post thaw motility (PTM) of spermatozoa in Group II (4 mmol cysteine, 44.5±1.7) as compared to the Control (27.5±1.5) and other treatments Group III (25.0±1.4), and IV (19.5±1.4) (Table 1). The percentage value of post thaw motility in present study is in agreement with Uyasal and Bucak (2007) who reported post thaw motility of 45.0±3.42 in frozen thawed semen in group containing 5 mmol cysteine. Michael *et al.* (2010) observed addition of L- Cysteine at the rate of 4 mmol provided better PTM values. On the contrary, Topraggaleh *et al.* (2013) reported greater PTM in the group containing 7.5 mmol cysteine in buffalo semen.

For the spermatozoa to maintain its motility mitochondrial integrity is must. Mitochondria cover the axosoma and accompanying dense fibres of the middle parts of sperm, which produce energy from ATP stored intracellularly (Garner and Hafez, 1993). The mitochondria present in the spermatozoa are the target of ROS because of oxidative phosphorylation (Thuwanut *et al.*, 2011). In agreement, Kadirvel *et al.* (2009) discovered a negative connection between mitochondrial membrane activity and ROS in fresh and frozen–thawed buffalo spermatozoa, Thus, the greater PTM in Group II could be related to mitochondria's increased antioxidant potential, playing a crucial role in sperm motility by acting as an energy source via oxidative phosphorylation (Koppers *et al.*, 2010). Based

on the present results it can be hypothesized that cysteine exerted cryoprotective influence on the integrity of mitochondria and axosoma thus improving the post thaw motility. However, the exact mechanism by which amino acids improve motility and membrane integrity is yet to be elucidated. On the other hand, the toxicity of greater amino acid concentrations during the freezing-thawing process has long been recognised in various earlier studies (Ahmad *et al.,* 2008).

Assessment of sperm viability is positively correlated with bull fertility rate. The mean percentage of spermatozoa viability was higher (60.9±1.9) in Group (4 mmol) as compared in the Control (51.4±2.2) and Group III (48.0±2.2, 8 mmol) and IV (45.8±2.1, 12 mmol) (Table 1).

Funahashi and Sano (2005) reported supplementation with 5 mmol glutathione or cysteine increased the survivability and functional integrity of swine spermatozoa during liquid storage at 10◦C for at least 14 days. This quoted study results are in agreement with in the current investigation. The positive effect of the cysteine could be assigned to the fact that it can quickly enter cells and be converted to taurine. This taurine can then be coupled with a fatty acid in the plasma membrane to generate acyl-taurine. This acyl-taurine can increase spermatozoa membrane surfactant characteristics and osmo-regulation (Esteves *et al*. 2007).

The integrity of spermatozoa plasma membrane influences post thawed spermatozoa viability and fertility (Mehmood et al., 2009). The mean percentages of plasma membrane integrity in different groups at post thaw stage were 45.6±2.9 in Control, 54.5±2.8 in Group (4 mmol), 46.3±2.6 in Group III (8 mmol) and 42.7±2.5 in Group IV (12 mmol) (Table 1). The results showed Group II had higher percentage of plasma membrane integrity as compared to other treatment groups (Group III and IV). In agreement, Funahashi and Sano (2005) observed improved viability and membrane integrity of boar semen cell during liquid storage when semen extender contained with 5 mmol cysteine. On the contrary, Topraggaleh *et al.* (2013) obtained good percentage of plasma membrane integrity at 7.5 mmol cysteine concentration than 5 mmol and 10 mmol in frozen thawed buffalo semen. However, in the present study, the greater percentage of plasma membrane integrity indicators was observed when cysteine was added at the rate of 4 mmol. The activity of trehalose and cysteine, which makes the plasma membrane less sensitive to cryo-damage during the freezing and thawing process, may be responsible for the improved cryopreserved semen quality (Memon *et al*., 2011). In the present study, it could be surmised thus that the principal reason for plasma membrane protection against ROS was due to reduction in superoxide anion caused by amino acid intake. Shoe and Zamiri (2008) observed that by increasing the flexibility of the plasma membrane, increased antioxidant concentrations make spermatozoa less sensitive to cryoinjury.

The metabolic substance from the poly unsaturated fatty acid membrane of spermatozoa plays a vital role in spermatozoa acrosome reaction (Tripodi *et al*., 2003). During process of freezing and thawing the changes like cryocapacitation and excessive production of radicals damage the acrosome intactness affecting the sperm fertility *in vivo* (Medeiros *et al*., 2002). Premature hyper-activation may cause spermatozoa's acrosome integrity to deteriorate, resulting in lower fertility rates (Michael *et al*., 2010). The mean value for acrosome membrane integrity at post thaw stage in different groups in the present study were 76.6±2.2 (Control), 83.7±2.0 in Group II (4 mmol), 75.1±2.6 in Group III (8 mmol) and 74.0±3.0 in Group IV (12 mmol) (Table 1). The mean value of acrosome integrity shows no significant difference among treatment groups, however higher percentage value was observed in semen extender supplemented with 5 mmol cysteine in boar semen cell during liquid storage (Funahashi and Sano, 2005). This study results are in agreement with present study. On the other hand, Topraggaleh *et al*. (2013) reported higher percentage of acrosome integrity in semen extender supplemented with 7.5 mmol of cysteine in buffalo. In contrast, in the present study the semen extender supplemented with 4 mmol cysteine showed better percentage of acrosome

Table 1: Effect of cysteine on semen quality parameters (SQP, Mean±SEM, n=24, %)

SQP	Control	Treatment			*P* value
	Gr I	Gr II	Gr III	Gr IV	
Post-thaw motility	27.5±1.5^{b}	44.5±1.7^{a}	25.0±1.4^{bc}	19.5±1.4^{c}	0.000
Viability	51.4±2.2^{b}	60.9±1.9^{a}	48.0±2.2^{b}	45.8±2.1^{b}	0.000
Acrosome integrity	76.6±2.2^{ab}	83.7±2.0^{a}	75.1±2.6^{ab}	74.0±3.0^{b}	0.033
Plasma membrane integrity	45.6±2.9^{ab}	54.5±2.8^{a}	46.3±2.^{6ab}	42.7±2.5^{b}	0.022
Sperm abnormality	19.2±0.5^{a}	14.2±1.1^{b}	19.5±0.8^{a}	21.8±0.9^{a}	0.000
DNA integrity	90.5±0.2^{b}	95.7±0.1^{a}	86.2±0.3^{c}	84.5±0.3^{d}	0.000

Means with dissimilar superscripts (a, b, c) in a row differ significantly (p<0.05)

integrity. Woelders *et al*., (1997) reported that by inserting itself into membrane phospholipids, trehalose in extenders is believed to modify membrane fluidity and maintain membrane integrity during dehydration conditions. In sperm cells diluted with Tris extender supplemented with a mixture of trehalose, cysteine, and hypotaurine, the integrity of the cell plasma membrane, outer and inner acrosome membranes, mitochondrial dense electron spaces, and nuclear content uniformity were retained.

For egg fertilization and subsequent embryonic development integrity of the spermatozoa chromatin is essential. Moreover, the intactness of the nucleic acid is one of the good indicators for successful cryopreservation; it shows preserved genetic material (Jerome and Srivastava, 2012; Rieso *et al*., 2012). In the present study, the mean value (per cent) of spermatozoa with intact acrosome at post thaw stage in different groups were 90.5±0.2 in Control, 95.7±0.1 in Group II (4 mmol), 86.2±0.3 in Group III (8 mmol) and 84.5±0.3 in Group IV (12 mmol) (Table 1). Topraggaleh *et al*. (2013) reported no significant difference in DNA integrity among different groups, with spermatozoa in groups supplemented with 5 mmol and 7.5 mmol cysteine showed numerically less (per cent) DNA damage in buffalo semen. Similar to this cited study, in the current investigation in the group extender supplemented with 4 mmol cysteine had higher percentage of DNA integrity. Bilodeau *et al*., (2000) observed that cryopreservation of bull spermatozoa drastically diminishes cellular glutathione levels by 78%.

The structural abnormalities of spermatozoa are mainly due to faulty spermatogenesis caused by disease and environmental condition, and improper semen handling procedures. In the present study, the mean values for spermatozoa abnormalities at post thaw stage in different groups were 19.2±0.5 in Control, 14.2±1.1in Group II (4 mmol), 19.5±0.8 in Group III (8 mmol) and 21.8±0.9 in Group IV (12 mmol) (Table 1). Above results show that the Group II had less abnormalities than the Control and other treatment groups (III and IV). Sarýözkan *et al*. (2009) reported 5 mmol cysteine supplemented to the semen extender produced low percentage of abnormalities as compared to the taurine and the Control in bull frozen thawed semen. This is in agreement with the current study. On the other hand, Tuncer et al. (2010) found no significant variation in spermatozoa morphology in frozen-thawed bull semen when extender was supplemented with 5 mmol, and 10 mmol cysteine and the Control. Results of this study find support from the observation of the reports from Eskiocak *et al*., (2005) observing the adverse linkage of cysteine concentration with fraction of spermatozoa with abnormalities. . The thiols group of cysteine helps in preserving the cytological parameters of spermatozoa, thereby decreasing the number of spermatozoa abnormalities.

Semen quality *vis-a-vis* reactive oxygen species at post thaw

The end product of the lipid peroxidation is the malondialdehyde (MDA) which is measured by the thiobarbituric acid assay (sanocka and Kurpisz, 2004). In the current study, the mean values of malondialdehyde were 0.7±0.1(fresh stage), 1.9±2.2 (Control), 1.3±2.2 (Group II, 4 mmol), 2.3±7.8 (Group III, 8 mmol), 2.5±3.4 (Group IV, 12 mmol) (Table 2, Figure 1).

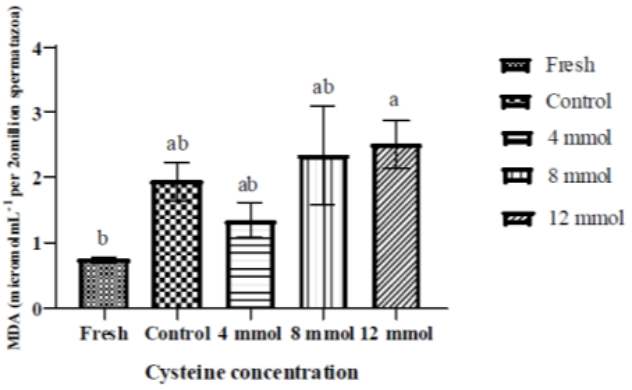

Fig. 1: Effect of cysteine on lipid peroxidation status (MDA molmL^{-1} per 20 million spermatozoa) at fresh and post thaw stage (Mean±SEM, n=24). Means with dissimilar superscripts (a, b, c) in a row differ significantly (p<0.05)

Table 2: Effect of cysteine on lipid peroxidation status (MDA molmL^{-1} per 20 million spermatozoa), Total anti-oxidant capacity (TAC, µmol), and intracellular reactive oxygen species (ROS) at fresh and post thaw stage (Mean±SEM, n=24)

SQP	Fresh stage	Control	Post thaw stage			*P* value
		Gr I	Gr II	Gr III	Gr IV	
LPO	0.7±0.1^{b}	1.9±2.2^{ab}	1.3±2.2^{ab}	2.3±7.8^{ab}	2.5±3.4^{a}	0.018
TAC	1283.8±46.7^{a}	783.8±66.1^{bc}	967.8±22.8^{b}	717.7±57.1^{c}	707.7±42.6^{c}	0.000
Intracellular ROS	NA	40.5±1.4^{b}	34.9±2.1^{b}	40.8±2.5^{b}	57.3±1.7^{a}	0.000

NA, Not estimated; Means with dissimilar superscripts (a, b, c) in a row differ significantly (p<0.05)

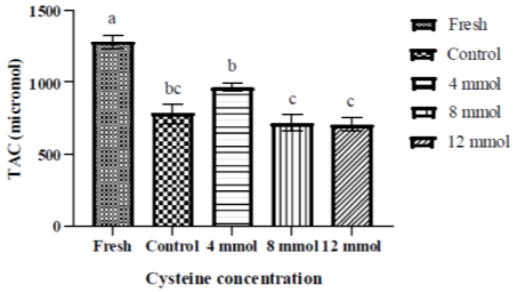

Fig. 2: Effect of cysteine on Total anti-oxidant capacity (µmol) at fresh and post thaw stage (Mean±SEM, n=24). Means with dissimilar superscripts (a, b, c) in a row differ significantly (p<0.05)

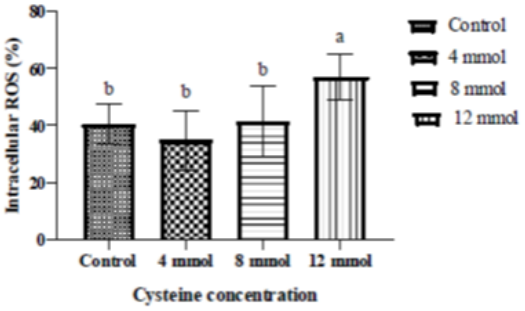

Fig. 3: Effect of cysteine on intracellular ROS (Mean±SEM, n=24, %). Means with dissimilar superscripts (a, b) in a row differ significantly (p<0.05).

The results show low MDA production in semen extender supplemented with the 4 mmol cysteine than other groups (Control, Group II, III, and IV). The level of the MDA production increased from fresh to post thaw stage in all the treatment groups in present study which is in agreement with results of Kadirvel *et al.* (2009). This could be attributed to the fact that buffalo spermatozoon membrane has rich polyunsaturated fatty acid and low-level antioxidant which makes them easily susceptible to oxidative stress following freezing thawing as compared to fresh spermatozoa (Trinchero *et al.*, 1990). The buffalo spermatozoa membrane contains rich polyunsaturated fatty acid making them more susceptible to LPO (Nair *et al.*, 2006). Furthermore, cysteine can be converted to taurine and acetyl–taurine to improve sperm membrane osmo-regulation and prevent lipid peroxidation (Kaeoket *et al.*, 2010). Since L-cysteine reduces lipid peroxidation, thus protecting spermatozoa from free radicals (Chatterjee *et al.*, 2001)

The seminal plasma contains large number of antioxidants (Bathgate, 2011) which help to protect the spermatozoa from oxidative stress, either produced by spermatozoa itself or the surrounding media. These antioxidants can be measured using ferric reducing/antioxidant power (FRAP) assay (Benzie & Strain *et al.,* 1996). In current study the mean value of total antioxidant capacity (µmol) were 1283.8±46.7 in fresh stage, 783.8±66.1in Control, 967.8±22.8 in Group I (4 mmol), 717.7±57.1in Group II (8 mmol), 707.7±42.6in Group III (12 mmol) (Table 2, Figure 2). In all groups, the mean values were significantly lower in the post-thaw stage compared to the fresh stage, which could be attributable to the use of antioxidants to neutralize the oxidants created during the cryopreservation process (Din *et al.*, 2018). Among the four groups, the TAC value was greater in Group II (4 mmol) than Control Group III and IV, a result which might be due to greater antioxidant potential of cysteine. Higher TAC value was observed in 4 mmol of cysteine group than Control and Group III, and IV which might be due to supplementation with targeted antioxidant. This helped to reduce ROS production from plasma membrane. Lower values of TAC in Group III and IV than control could be attributed to the amino acid toxicity. Cysteine, Trehalose, and hypotaurine have a beneficial effect on cryopreserved buffalo spermatozoa because of their capacity to preserve bio-membrane stability, scavenge ROS, and protect sperm cells from hazardous oxygen metabolites that cause lipid peroxidation (Badr *et al.*, 2014).

The production of excess ROS causes oxidative stress which is accountable for poor post thaw semen quality during cryopreservation process (Bucak *et al.*, 2008, Ngau *et al.,* 2020). ROS generates physical and chemical stress on the sperm membrane, as well as causing lipid peroxidation and sperm degradation (Mustapha, 2017). The per cent production of intracellular ROS was 40.5±1.4 (Control), 34.9±2.1 (Group I), 40.8±2.5 (Group II), Group III, 57.3±1.7in Group IV (Table 2, Figure 3). The present study showed significantly reduction in intracellular ROS in spermatozoa of the groups supplemented with the 4 mmol cysteine. However, Topraggaleh *et al.* (2013) reported supplementation of 7.5 mmol of cysteine significantly reduced intracellular ROS in buffalo spermatozoa. The cysteine is an antioxidant which enhances the production or synthesis of glutathione (GSH). This in turn play a vital role as endogenous antioxidant involved in maintaining the tissue antioxidant–prooxidant balance (Andreea *et al.*, 2010). In agreement, Mata-Campuzano *et al.* (2012) reported reduction in the intracellular ROS when adding antioxidants such as N-acetyl-cysteine applied after 4 h of incubation in ram semen.

CONCLUSIONS

It is concluded from the study that supplementation of cysteine at 4 mmol concentration can improve the post thaw semen quality parameters of buffalo spermatozoa, though cysteine addition at 8 or 12 mmol either does not impart

any beneficial effects or has adverse effect and may cause poor freezability of buffalo spermatozoa. Thus, cysteine can serve as a cost-effective and efficient agent to alleviate heat-stress mediated spermatozoa damage

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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