RESEARCH ARTICLE

Mitigation of Cryopreservation Induced Changes in Murrah Buffalo *(Bubalus bubalis)* spermatozoa using L-Cysteine Hydrochloride

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Abstract

This investigation was carried out with the objective of finding improvements in the quality of frozen semen from buffalo bulls with the incorporation of cysteine as an additive in tris dilutor. Thirty-six ejaculates from six Murrah buffalo bulls of 6-8 years of age group were collected in sterilized artificial vagina to evaluate the effect of additives at the post-dilution and post-thaw stages. The semen sample was extended with Tris-Citric-acid-Fructose-Egg-Yolk--Glycerol (TFYG) extender and was divided into two groups: Group I without any additive served as control (C) group, while Group II, extender supplemented with L-cysteine hydrochloride (1.0 mM) served as treatment (T) group. Progressive sperm motility, live spermatozoa, sperm abnormalities, and enzyme leakage (AST and ALT) were evaluated at both post-dilution and post-thaw stages. There was significant ($p<0.01$) improvement in progressive motility and live spermatozoa, as well as a significant ($p<0.01$) decrease in sperm abnormalities and enzyme leakage (AST and ALT) in the cysteine-treated group compared to the control group. The per cent sperm abnormalities had significant negative correlations with sperm motility and viability, and positive correlations with seminal AST and ALT activity at both on dilution and post-thaw stage of semen. It was concluded that the addition of 1.0 mM L-cysteine hydrochloride to the Tris extender significantly ($p<0.01$) improves the quality of cryopreserved bubaline semen.

Key words: Additives, Cryopreservation, L-cysteine hydrochloride, Murrah semen.

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Introduction

Cryopreservation causes oxidative stress, which reduces the viability and fertility of buffalo bull sperm (Kumar *et al.,* 2011) because of higher lipid peroxidation levels in the plasma membrane, oxidative stress during the freeze-thawing process accelerates the production of reactive oxygen species. Buffalo bull spermatozoa contain more polyunsaturated phospholipids than cattle spermatozoa, making them more susceptible to freeze-thawing stress (Andrabi, 2009). The conception rate with frozen-thawed buffalo semen is only about 33% (Bhosrekar *et al.,* 2001), which could be attributed to a decrease in motility, damaged acrosomes, and altered plasma membrane integrity caused by cryodamage during the freezing process (Rasul *et al.,* 2001). During cryopreservation, there is an imbalance between ROS and natural antioxidants, resulting in oxidative stress. As a result, adding antioxidants to sperm extenders is required to reduce the effects of oxidative stress on spermatozoa during cryopreservation (Andrabi *et al.,* 2008). L-Cysteine hydrochloride, a non-enzymatic antioxidant (Bilodeau *et al.,* 2001), a precursor of intracellular glutathione, has expressed the ability to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both *in-vivo* and *in-vitro* and insulating the membrane lipids and proteins due to implied radical scavenging properties (Memon *et al.,* 2011) and prevents apoptosis, freezing-thawing damages, and ROS production during low-temperature storage (Bilodeau *et al.,* 2001). Outcomes of spermatozoa cryopreservation with cysteine hydrochloride in extender have been reported in Akkaraman rams (Bucak *et al.,* 2007) and Nili-Ravi buffalo (Wadood *et al.,* 2015). Moreover, oral supplementation of N-acetyl cysteine has been reported to improve the morphologic features of spermatozoa and oxidative/antioxidant status in infertile

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males (Janntifar *et al.,* 2019). Keeping in view the importance of buffalo as an important dairy animal for the Indian livestock economy and the need for scientific intervention in the cryopreservation process of buffalo semen, the present study was conducted to evaluate the effect of L-Cysteine hydrochloride as an additive in extender on freezability of Murrah buffalo bull spermatozoa.

Material and Methods

Semen samples were collected from six healthy Murrah buffalo bulls aged 4-8 years, weighing 450-700 kg, which were reared at the Deep Frozen Semen Lab, College of Veterinary Sciences & Animal Husbandry, ANDUAT, Kumarganj, Ayodhya, Uttar Pradesh (India). The bulls were maintained in a similar environment and subjected to identical conditions of management and feeding and used for study following approval by the Institutional Animal Ethics Committee. A total of thirty-six ejaculates, collected in a sterilized artificial vagina, were used to evaluate the effect of L-Cysteine hydrochloride on semen cryopreservation. After collection, the semen samples were immediately transferred to a water-bath maintained at 35°C and kept under laminar airflow. Semen samples were evaluated for progressive sperm motility, sperm concentration (Accucell Photometer IMV France), live sperm per cent and sperm abnormalities (eosin-nigrosin staining) as per the standards procedures, and the activity of aspartate and alanine aminotransferases (AST-ALT) were estimated by BEACON $BAuto^{200}$ analyzer with commercially available kits.

The selected ejaculates (having >70% visual motility) were diluted in Tris-Citric-acid-Fructose-Egg-Yolk-Glycerol (TFYG) extender. The ejaculates after dilution were split into two parts: Part 1: Diluted in Tris-Egg-Yolk-Citric-acid-Fructose-Glycerol without additive served as Control (C), and Part 2: Diluted in TFYG with addition of 1.0 mM L-cysteine hydrochloride served as treatment (T).

Diluted semen was filled-in French mini straws and sealed with PVA powder. These straws were kept for equilibration at 4°C for 4 h. Subsequently, the semen straws were then cleaned, wiped on filter paper, and spread evenly over the rack. The rack along with the straws was kept in a programmable biofreezer and frozen as per standard protocol, and then plunged into liquid nitrogen (-196°C) and further stored for 48 hours. After being thawed at 37°C for 30 sec, each sample was examined for post-thaw motility, viability and morphology (% live count and abnormal sperm) using the standard methods.

All the chemicals, including L-cysteine hydrochloride, used in the experiment were procured from Merck, USA. Data were presented as the mean and standard error of the mean (SEM). The data were analyzed statistically using one-way ANOVA, Duncan's NMRT, and Pearson's correlation coefficients among various seminal attributes were determined.

Results and Discussion

The results of the trial conducted to evaluate the effect of a 1.0 mM concentration of L-cysteine hydrochloride on the different seminal attributes (sperm progressive motility, livability, abnormality, and AST & ALT) are summarized in Table 1.

The post-thawed seminal parameters such as sperm motility and sperm livability showed a significant ($p<0.05$) increase, whereas total sperm abnormality and enzyme leakage (AST and ALT) were significantly ($p<0.05$) lower in the cysteine hydrochloride-treated group as compared to the control group. The per cent initial motility of spermatozoa in post-diluted semen was recorded as 70.11±0.17 and 72.42±0.19, whereas post-thaw sperm motility was recorded as 45.39±0.33 and 58.17±0.36 in the control (C) and cysteine-treated group (T), respectively. More than 50% of spermatozoa are usually injured by the cryopreservation process (Watson, 1995). The data revealed that cysteine significantly improves post-thaw motility, which was in full agreement with the observations of Funahashi and Sano (2005). Bilodeau *et al.* (2001) demonstrated that thiol radical-containing amino acids such as glutathione, cysteine, N-acetyl cysteine, and 2-merkeptoethenol prevent hydrogen peroxide-mediated loss of sperm motility in frozen-thawed bull semen. The progressive sperm motility of post-diluted and post-thaw semen was significantly ($p<0.01$) positively correlated with sperm livability (r=0.77 & 0.84), whereas it was negatively correlated with per cent sperm abnormality (r=-0.70 & -0.75), seminal plasma AST (r=-0.75 & -0.68) and ALT (r=-0.89 & -0.70, respectively) in the cysteine (T) treated group (Table 2). These correlations were similar to the observations of Srivastava (2011) and Saurabh (2021) in Murrah bulls. Regarding the addition of cysteine to the semen extender, the present study demonstrated that cysteine exhibited a positive impact on frozen sperm parameters and enhanced the biochemical characteristics. These results were in agreement with several studies performed on cow bull (Sariozkan *et al.,* 2009) semen. The positive effect of cysteine over spermatic parameters is due to cysteine being an amino acid and a precursor in the production of intracellular glutathione (GSH), which neutralizes the reactive oxygen species and catalyzes hydrogen or other superoxide detoxification (Memon *et al.,* 2011). Moreover, it acts as a cofactor of glutathione peroxidase to knock-down hydrogen peroxide (Andrea *et al.,* 2009).

The mean (± SE) per cent viability of post-diluted semen in the control (C) and cysteine-treated (T) extender was observed as 83.53±0.45 and 84.56±0.32, whereas the values in post-thawed semen were 66.25±0.54 and 73.06±0.51, respectively (Table 1). The observation of post-diluted semen was similar to the finding recorded by Felipe-Perez *et al.* (2008), whereas post-thaw viability was higher than their findings. The per cent live count of post-diluted and post-thaw semen was significantly ($p<0.01$) and negatively correlated with sperm abnormality (r=-0.66 & -0.68), seminal plasma AST (r=-0.56 & -0.63) and ALT (r=-0.74 & -0.73), respectively,

(Table 2). These correlations align with the findings of Kumar (2014). The incorporation of cysteine HCl significantly (p<0.05) enhanced the post-thaw live sperm count among the bulls. Cysteine is an antioxidant that protects the sperm cell from oxidative stress (Ansari, 2011). Cysteine can directly neutralize the free radicals and/or act through a gluthione-mediated cellular pathway. Supplementation of cysteine in extender resulted in better post-thaw semen quality (Ansari, 2011).

The overall per cent (mean ± SE) sperm abnormality in post-diluted semen recorded was 8.08±0.13 and 8.11±0.12, whereas in post-thaw semen it was 15.86±0.19 and 12.11±0.20 in the control and cysteine treated group, respectively. The addition of cysteine HCl significantly (p<0.05) reduced the post-thaw sperm abnormalities. The observation of post-diluted semen was comparable with the findings of Hirabhai *et al.* (2022), whereas post-thaw sperm abnormality was comparable to that of Saurabh (2021). Oxidative stress causes increased reactive oxygen species (ROS) production in animals, which causes cellular damage via structural and functional changes and may result in sperm abnormalities (Valeanu *et al.,* 2015). The per cent sperm abnormality of post-diluted and post-thawed semen was significantly (p<0.01) weakly correlated with seminal plasma AST (r=-0.72 & 0.36) and ALT (r=0.55 & 0.44) (Table 2). Cysteine is a sulfur-containing amino acid found naturally in seminal plasma and sperm nucleic acid. It acts as an antioxidant directly and indirectly through intracellular antioxidants that protect against ROS-mediated deleterious effects (Topraggaleh *et al.,* 2014). Cysteine reinforces glutathione levels because it is a precursor of intracellular glutathione biosynthesis. It is worth noting that a significant decrease in the level of endogenous antioxidants was observed during the freeze-thawing of bovine sperm (Beheshti *et al.,* 2011).

The overall activities of alanine transaminase (ALT) in seminal plasma of post-diluted semen were recorded as 23.92±0.17 and 21.67±0.23 µmole/L, whereas post-thaw alanine transaminase (ALT) activity recorded was 30.14±0.40 and 26.31±0.25 µmole/L in Control and cysteine-treated groups, respectively. A significantly lower ALT activity was observed in the cysteine-treated group as compared to the control. A similar ALT activity was reported by Srivastava (2011), whereas higher enzymatic activity was recorded by Rasul (2000). The ALT activity in plasma of post-diluted and post-thawed semen was significantly (p<0.01) and negatively correlated with sperm motility (r=-0.89 & -0.70), live sperm count (r=-0.74 & -0.73) and positively correlated with sperm abnormality (r=0.55 & 0.44) (Table 2).

The overall activity of aspartate transaminase (AST) in seminal plasma of control and cysteine-treated groups at post-dilution was recorded as 105.90±2.20 and 90.39±0.87 µmole/L, whereas post-thaw aspartate transaminase (AST) was recorded as 155.0±1.34 and 132.10±1.79 µmole/L, respectively. Our findings were in accordance with the previous report of Patel *et al.* (2016), but lower than Chaudhari *et al.* (2015) and higher than El-Nagar (2017). Our present overall findings on sperm quality parameters, HOST and

Table 1: Effect of L-cysteine hydrochloride (1.0 mM) supplementation in Tris extender on sperm quality parameters and enzyme leakage during cryopreservation of Murrah bulls semen

Stages	Treatment	Seminal attributes			Enzymatic constituents	
		Motility (%)	Livability (%)	Sperm abnormality (%)	ALT (µmole/L)	AST (µmole/L)
Post-dilution	Control (Tris)	70.11±0.17^{a}	83.53±0.45^{a}	8.08±0.13^{a}	23.92±0.17^{b}	105.90±2.20^{b}
	Tris + L-cysteine HCl 1.0 mM (T)	72.42±0.19^{b}	84.56±0.32^{b}	8.11±0.12^{a}	21.67±0.23^{a}	90.39±0.87^{a}
Post-thaw	Control (Tris)	45.39±0.33^{A}	66.25±0.54^{A}	15.86±0.19^{B}	30.14±0.40^{B}	155.0±1.34^{B}
	Tris + L-cysteine HCl 1.0 mM (T)	58.17±0.36^{B}	73.06±0.51^{B}	12.11±0.20^{A}	26.31±0.25^{A}	132.1±1.79^{A}

AST-ALT= Asparate and Alanine amino-transferases, Mean ± SE values bearing different superscript in a column within the stage (a, b / A, B) differ significantly (P<0.05).

Table 2: Correlation coefficients (r=values) among different seminal attributes in post dilution and post-thawed semen of Murrah bulls

Stages	Parameters	Sperm motility	Live sperm	Abnormal sperm	Seminal ALT
Post-dilution	Live sperm count	0.77**	--	--	--
	Abnormal sperm	-0.70**	-0.66**	--	--
	Seminal ALT	-0.89**	-0.74**	0.55**	--
	Seminal AST	-0.75**	-0.56**	0.72**	0.58**
Post-thaw	Live sperm count	0.84**	--	--	--
	Abnormal sperm	-0.75**	-0.68**	--	--
	Seminal ALT	-0.70**	-0.73**	0.44*	--
	Seminal AST	-0.68**	-0.63**	0.36*	0.72**

*Significant at 5%, ** Significant at 1%

enzyme activity of buffalo semen pre- and post-freezing using L-cysteine concurred well with the findings of Verma *et al*. (2023) using methionine as a semen additive @ 2.0 and 2.5 mM in Murrah buffalo semen cryopreservation. AST and ALT are essential for metabolic processes that provide energy for the survival, motility, and fertility of spermatozoa, and these transaminases' activities in semen are good indicators of semen quality because they measure sperm membrane stability (Asadpour, 2012). High concentration of transaminase enzyme in the extracellular fluid is due to sperm membrane damage and ease of leakage of enzymes from spermatozoa (Gundogan, 2006). The addition of antioxidants in extenders aimed to reduce oxidative stress and prevent cold shock and cryo-injury to spermatozoa. During freezing, the spermatozoa membrane is damaged, resulting in the leakage of enzymes. Our study showed that the addition of cysteine hydrochloride to freezing extender prevents enzyme leakage through the action of membrane stabilizer, as stated by Matilde *et al*. (2005). Moreover, cysteine has a cryoprotective effect on the functional integrity of the axosome and mitochondria, improving post-thawed sperm motility in many species, *i.e.,* ram (Uysal and Bucak, 2007) and bull semen (Bilodeau *et al.,* 2001).

Conclusion

Based on the results of this study, it can be concluded that the addition of a dose of 1.0 mM L-cysteine hydrochloride in the Tris-Citric Acid-Fructose-Egg-Yolk-Glycerol diluent has a significant effect ($p<0.05$) on post-thaw sperm motility, sperm viability, sperm abnormalities, and AST and ALT of buffalo semen. L-cysteine hydrochloride in extender provides better protection to spermatozoa during dilution, freezing, and thawing stresses than the control extender.

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