Journal of Environmental Biology
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

JEB®

Original Research Paper
Volume 46 Issue 3 May 2025 pp. 329-335
DOI: http://doi.org/10.22438/jeb/46/3/MRN-5402

J. Environ. Biol., 2025
© Triveni Enterprises, Lucknow (India)
Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Methionine supplementation improves freezability of Jamnapari Buck semen

K. Alam[1], S. Srivastava[1], S.K. Verma[1], A. K. Verma[1], B. Singh[1], Saurabh[2], R. Kumar[1]*, R. Kumar[1], A. Jaiswal[3], R.K. Verma[2], A. Alam[4], J. Singh[5], R.R. Singh[6], P. Kumar[7], H.C. Verma[8] and N. Singh[1]

[1]Department of Veterinary Gynaecology & Obstetrics, [2]Department of Veterinary Clinical Complex, [3]Department of Livestock Production Management, [4]Department of Veterinary Medicine, [5]Department of Animal Genetics & Breeding, [6]Department of Soil Science, College of Agriculture, [7]Department of Veterinary Physiology & Biochemistry, [8]Department of Veterinary and Animal Husbandry Extension Education, College of Veterinary Science & Animal Husbandry, AcharyaNarendra Deva University of Agriculture and Technology, Kumarganj-224 229, India

Received: 28 May 2024 **Revised:** 18 September 2024 **Accepted:** 06 January 2025

***Corresponding Author Email:** drrajesh25@gmail.com ***ORCiD:** https://orcid.org/0000-0001-5751-7359

Online Copy

Abstract

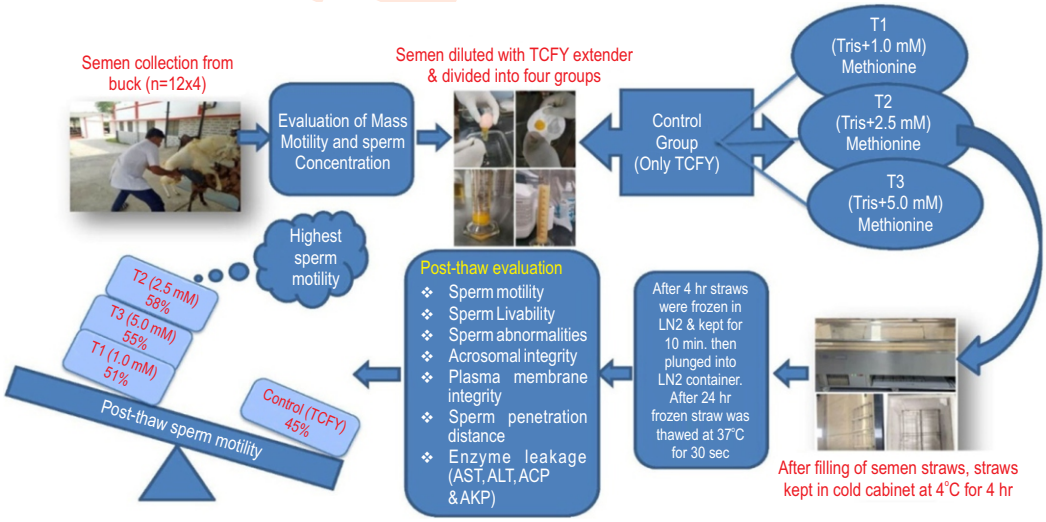
Aim: The study aimed to assess the effect of methionine supplementation as a semen additive to augment the freezability of Jamnapari buck semen.

Methodology: A total of forty-eight ejaculates were collected from four Jamnapari bucks (n=12x4) twice a week using an artificial vagina from October to November 2023. The sperm concentration was estimated using a photometer and semen was diluted with tris-citrate-fructose-yolk extender and divided into four equal aliquots (control, T1, T2 and T3) and methionine was added @ 0, 2, 2.5, and 5.0 mM in C, T1, T2 and T3, respectively. The post-diluted and frozen-thawed semen was evaluated for sperm motility, viability, acrosomal integrity, sperm abnormalities, sperm plasma membrane integrity, sperm penetration distance, and enzyme leakage (AST, ALT, ACP and AKP).

Results: Methionine applied @ 2.0 and 5.0 mM in the extender seemed to be less effective at maintaining sperm structural integrity and functionality in comparison to the 2.5 mM treatment. The inclusion of 2.5 mM methionine has significantly enhanced the freezability of Jamnapari buck semen.

Interpretation: In conclusion, methionine in tris-citrate-fructose-yolk extender can improve the sperm quality and fertility potential of post-thawed spermatozoa of Jamnapari buck.

Key words: Cryopreservation, Jamnapari bucks, Methionine, Semen



How to cite: Alam, K., S. Srivastava, S.K. Verma, A.K. Verma, B. Singh, Saurabh, R. Kumar, R. Kumar, A. Jaiswal, R.K. Verma, A. Alam, J. Singh, R.R. Singh, P. Kumar, H.C. Verma and N. Singh: Methionine supplementation improves freezability of Jamnapari Buck semen. *J. Environ. Biol.*, **46,** 329-335 (2025).

Introduction

Cryopreservation is an efficient technique for the long-term preservation of spermatozoa; nonetheless, it may induce freezing damage (Andrabi, 2009). Spermatozoa undergo considerable damage due to oxidative stress during the freeze-thawing process (Fayyaz *et al.,* 2017). The viability and fertilization potential of spermatozoa are diminished by a series of environmental alterations that transpire during the freeze-thawing process (Oehninger *et al.,* 2003). Moreover, elevated levels of reactive oxygen species (ROS) in semen negatively affect both the sperm's ability to move and the capacity to fertilize (Taylor, 2001). Buck sperm is prone to lipid peroxidation due to the presence of a significant amount of polyunsaturated fatty acids (Shah *et al.,* 2017). Subsequently, lipid peroxidation results in the generation of more reactive oxygen species. The addition of antioxidants may help mitigate the effect of reactive oxygen species-induced oxidative stress occurring during sperm storage (Michael *et al.,* 2009). Thus, it is crucial to have a robust exogenous antioxidant system in place to prevent cryo injuries and lipid peroxidation (Irvine, 1996). The inclusion of antioxidants in cryo-diluent is beneficial in enhancing the quality of spermatozoa after freezing and thawing (Rao *et al.,* 2013; Dewry *et al.,* 2015). The use of antioxidants is employed by researchers as a mean to counteract the negative impacts of elevated levels of ROS in semen freezing extenders (Ghadimi *et al.,* 2024).

Methionine serves as a precursor of amino acid for the synthesis of glutathione. The antioxidant properties of methionine in preventing oxidative damage have been well-documented in several tissues through methionine therapy. Furthermore, various studies have demonstrated that sulfur-containing compounds, such as methionine, can bind to lead and eliminate it from tissues, thereby playing a crucial part in detoxification (Reed, 1990; Patra *et al.,* 2001). Indeed, methionine has important roles in the antioxidant defence system as it readily reacts with oxidants to produce methionine sulfoxide (Levine *et al.,* 1999). Furthermore, it has been demonstrated that methionine maintains bull spermatozoa's normal morphology and membrane integrity (Bucak *et al.,* 2010a). Recently, it has been documented that adding methionine to the extender can improve the motility of frozen-thawed Murrah bull sperm (Verma *et al.,* 2023). To the best of our knowledge, no previous studies have explored the role of methionine in the cryopreservation extender in mitigating cryodamage to Jamnapari goat semen. Therefore, this study was conducted to determine the effect of supplementation of the freezing extender with the antioxidant methionine on sperm motility, morphology, sperm abnormalities, plasma membrane integrity and seminal plasma enzyme leakage in post-thawed Jamnapari buck semen.

Materials and Methods

Animals and semen collection: This study utilized semen samples obtained from four adult Jamnapari bucks (2–3 year old) with superior genetic merit and fertility capacity. The bucks were raised at the Deep Frozen Semen Laboratory, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, India. They were kept under standard nutritional and managerial conditions (National Research Council, 2007). Forty-eight ejaculates were harvested from four sexually mature Jamnapari bucks (n=12x4) twice per week using an artificial vagina (AV) from 1st October to 30th November 2023; immediately after collection, the ejaculates were placed in a water bath at 35°C.

Preparation of extender: To prepare extender and other analytical work, analytical-grade reagents and double-glass distilled water were utilized. Tris-citrate-fructose-yolk extender (TCFY) (Gangwar *et al.,* 2015) was used as a diluter. In short, 1.902 g citric acid, 3.602 g tris-(Hydroxymethyl amino methane, Sigma-Aldrich), 1.00 g fructose, 6 ml glycerol (Merck, USA), and 1000 IU ml^{-1} Penicillin G-sodium were added to the final volume of TCFY extender, maintaining a pH of 6.8, made up to 100 ml by adding distilled water. To this, 10% egg yolk (v/v) was added at the time of use.

Semen extension, freezing and thawing: Ejaculates show visual motility greater than 80% and a concentration exceeding 2.5x10^{9} spermatozoa ml^{-1} (Accucell photometer, IMV, France) were diluted with a tris-citrate-fructose-yolk extender. Diluted semen was partitioned into four equal aliquots, with methionine subsequently added at concentrations of 0, 2, 2.5, and 5.0 mM to the control (C) and treatment groups T_1, T_2, and T_3, respectively. Diluted semen samples were filled into 0.25 ml French straws, sealed by the ISEVO machine (IMV Technologies), and equilibrated at 4°C for 4 hr. After equilibration, the straws were frozen in liquid nitrogen vapour, 2.5 cm above the liquid nitrogen, for 10 min and plunged into the liquid nitrogen for storage (Ranjan *et al.,* 2015; Gangwar *et al.,* 2015). After storing for 24 hr, the frozen straws were thawed at 37°C for 30 sec in a water bath for microscopic evaluation (Sariozkan *et al.,* 2010).

Evaluation of semen: Semen samples were evaluated for pre-freeze (on dilution) and post-thaw sperm motility, viability, and morphology using the established protocols of Hancock (1951) and modified by Ranjan *et al.* (2009). The hypo-osmotic swelling test was conducted following the protocol outlined by Ranjan *et al.* (2017). The acrosomal integrity of spermatozoa was assessed using Giemsa stain (Watson, 1975; Ranjan *et al.,* 2014). The polyacrylamide gel penetration test was conducted and evaluated using the grading system developed by Lorton *et al.* (1981). The seminal plasma was separated from freshly diluted and frozen-thawed samples using centrifugation at 3000 rpm for 20 min. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (AKP) were determined using commercially available standard kits (CliniQuant^{TM} micro) and a Merilizer (Meril Diagnostic Pvt. Ltd.).

Chemicals: The semen additive methionine (USB, Corporation Cleveland, OH, USA) and other chemicals (tris, citric acid,

fructose and glycerol) used in this study were obtained from Merck, KGaA 64271 Darmstadt, Germany.

Statistical analysis: The data obtained were presented as means ± SE. Means were analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison Test to determine significant differences in all the parameters between all groups, *i.e.,* control and treatment, using the Graph Pad Prism 5 software. The significance level was determined at $p < 0.05$.

Results and Discussion

The cryo-protective effect of methionine supplementation (2.0, 2.5 and 5.0 mM) and tris-citrate-fructose-yolk (TCFY) on different seminal attributes of Jamnapari buck semen after freezing/thawing process is depicted in Table 1. The results showed that the progressive motility of frozen-thawed spermatozoa significantly ($p<0.05$) increased when tris-citrate-fructose-yolk (TCFY) extender was supplemented with 2.0 mM, 2.5 mM and 5.0 mM methionine as compared to the control extender. A significantly ($p<0.05$) higher post-thaw progressive motility was observed in the methionine-treated group (T_2) as compared to other methionine-treated groups (T_1 and T_3). The results indicated a statistically significant ($p<0.05$) improvement in the number of viable spermatozoa and a reduction in sperm abnormalities on application of methionine (2.0 mM, 2.5 mM and 5.0 mM) to tris-citrate-fructose-yolk extender as compared to the control group. Moreover, there was a significant ($p<0.05$) difference observed in post-thaw sperm acrosomal integrity between the control and treatment groups. However, a non-significant ($p<0.05$) difference was observed between the treatment groups T_1 and T_2.

The sperm plasma membrane contains a high amount of polyunsaturated fatty acids, making it vulnerable to per oxidative damage. This damage causes a reduction in membrane integrity and decreased sperm motility ultimately leading to reduced fertility due to reactive oxygen species during aerobic incubation (Griveau *et al.,* 1995). The freezing procedure adversely affect sperm viability which ultimately reduce fertility (Verma *et al.,* 2023). According to Watson (2000), about 50% of the mobile sperm cells survive during cryopreservation under favourable conditions can be mitigated by adding antioxidants to the semen extender before storage (Michael *et al.,* 2009). Methionine readily crosses the cell membrane, increasing intracellular glutathione production in vivo (Reed, 1990; Patra *et al.,* 2001). This event may have a cryoprotective effect on the functional integrity of the membrane and cytoplasmic components of sperm cells, including the axosome and mitochondria, which are related to sperm motility (Coyan *et al.,* 2010). Moreover, Tuncer *et al.* (2010) observed that Angora buck semen incorporated with methionine @ 2.5 and 5 mM, showed improved post-thaw sperm motility (63.6 ± 7.0% and 63.4 ± 3.1%, respectively) in comparison to control following the freeze-thawing process. Moreover, Abdulkareem *et al.* (2020) observed that the addition of methionine to a bull semen extender resulted in significantly higher initial motility, live sperm count, and acrosomal integrity ($P \leq 0.05$) compared to the control group (extender without additives).

In the present study, there were significant ($p>0.05$) differences observed in the sperm plasma membrane integrity and sperm penetration distance (mm/h) of the treatment groups (T_1, T_2, and T_3) as compared to the control group (Table 1). Plasma membrane functioning and integrity are important sperm indicators when assessing sperm quality because an intact sperm plasma membrane is a major barrier to sperm cell survival (Makarevich *et al.,* 2010). It also plays an important role in sperm metabolism, acrosome reaction, and capacitation (Forouzanfar *et al.,* 2013). The results also showed that methionine treatment (2.0 mM, 2.5 mM and 5.0 mM) significantly ($p<0.05$) decreased the sperm abnormalities (8.83±0.28, 6.65±0.30 and 9.29±0.44%) than control group (10.92±0.29%). Similarly, seminal plasma enzyme leakage (AST, ALT, ACP and AKP) also declined in frozen spermatozoa as compared to control.

Table 1: Effect of methionine supplementation in TCFY extender on sperm quality parameters during cryopreservation of Jamnapari Buck semen

Stages	Additive concentrations (mM)	Progressive motility (%)	Sperm livability (%)	Acrosomal integrity (%)	Sperm abnormality (%)	Plasma membrane integrity (%)	SPD (mm/h)
Post-diluted	Control ©	80.54 ± 0.56^{a}	82.69 ± 0.71^{a}	85.61 ± 0.73^{a}	5.83 ± 0.28^{d}	63.27 ± 0.87^{a}	31.67 ± 0.59^{a}
	2.0 mM (T_1)	82.56 ± 0.46^{b}	84.59 ± 0.60^{b}	87.60 ± 0.73^{bc}	3.87 ± 0.27^{b}	65.69 ± 0.94^{b}	33.90 ± 0.63^{b}
	2.5 mM (T_2)	84.54 ± 0.95^{d}	87.10 ± 0.80^{d}	89.56 ± 0.72^{d}	2.43 ± 0.20^{a}	67.58 ± 0.92^{d}	35.81 ± 0.61^{d}
	5.0 mM (T_3)	83.54 ± 0.66^{c}	85.59 ± 0.58^{c}	88.63 ± 0.73^{cd}	4.81 ± 0.29^{c}	66.69 ± 0.14^{c}	34.88 ± 0.62^{c}
Post- thaw	Control ©	45.54 ± 0.62^{A}	57.46 ± 0.72^{A}	65.48 ± 0.73^{A}	10.92 ± 0.29^{D}	43.67 ± 0.70^{A}	23.81 ± 0.61^{A}
	2.0 mM (T_1)	51.52 ± 0.66^{B}	71.67 ± 0.72^{C}	77.81 ± 0.70^{BC}	8.83 ± 0.28^{BC}	51.69 ± 0.72^{B}	28.80 ± 0.60^{B}
	2.5 mM (T_2)	58.50 ± 0.64^{D}	73.69 ± 0.70^{D}	80.82 ± 0.75^{D}	6.65 ± 0.30^{A}	54.81 ± 0.71^{D}	31.85 ± 0.63^{C}
	5.0 mM (T_3)	55.54 ± 0.68^{C}	70.65 ± 0.73^{B}	77.85 ± 0.74^{C}	9.29 ± 0.44^{C}	53.88 ± 0.69^{C}	30.87 ± 0.62^{D}

SPD= sperm penetration distance in polyacrylamide gel, AST-ALT= Asparate and Alanine amino-transferases. Mean ± SE (n=48) values bearing different superscript within the stage (a, b, c / A, B, C) in a column differ significantly ($P<0.05$).

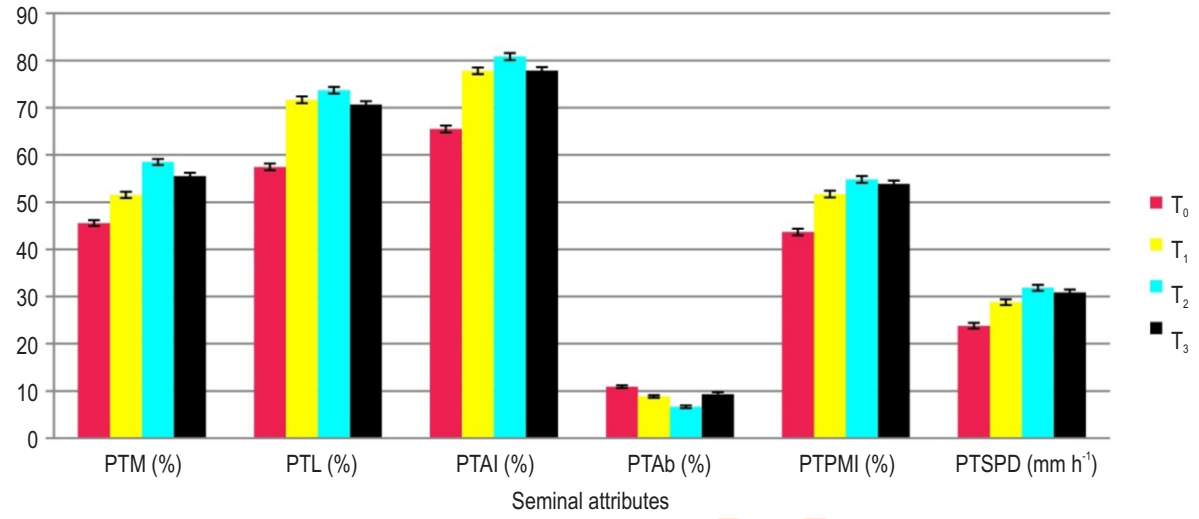


Fig. 1: Variation in overall mean ($p<0.05$) of progressive motility, sperm livability, acrosomal integrity, sperm abnormalities, plasma membrane integrity and sperm penetration distance by vanguard spermatozoa in frozen Jamnapari goat semen.

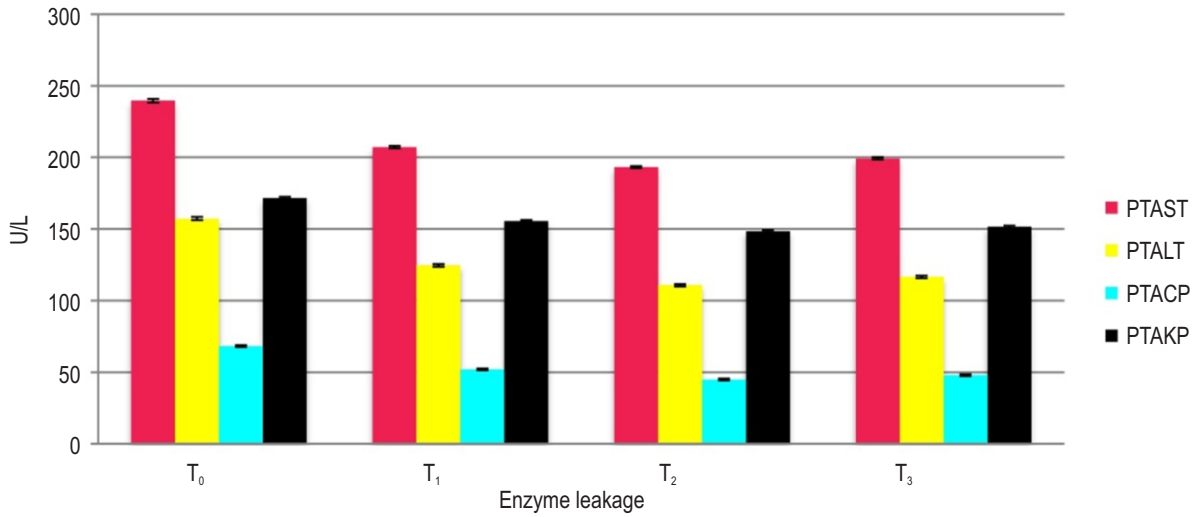


Fig. 2: Variation in overall mean ($p<0.05$) of seminal plasma enzyme leakage such as AST-ALT= Asparate and Alanine amino-transferases and ACP-AKP= acid and alkaline phosphatase in frozen Jamnapari goat semen.

Moreover, the lowest extracellular enzyme leakage was observed in the extender supplemented with 2.5 mM methionine (T_2) as well as other methionine-treated groups (T_1 and T_3) than control group (Table 2, Fig. 2). Seminal plasma is a complex mixture of secretions from several accessory sex glands, rich in chemicals that influence sperm fertilization ability (Turner and McDonnell, 2003). The enzyme AST-ALT activities in seminal plasma are effective predictors of sperm quality because they represent sperm plasma membrane stability, and increased enzyme activity implies enzyme leakage caused by reduced membrane stability (Gundogan, 2006). Furthermore, AST is required for the metabolic processes of spermatozoa, supplying energy for survival, motility and fertility (Perumal *et al.,* 2011).

Spermatozoa possess antioxidant mechanisms, such as glutathione (GSH), glutathione peroxidase and superoxide dismutase (SOD), in the semen to safeguard against oxidative stress-induced damage (Waisum *et al.,* 2006; Fernandez-Santos *et al.,* 2007). The plasma membranes of mammalian spermatozoa experience spontaneous peroxidation due to excessive generation of reactive oxygen species during cryopreservation. As a result, the lipid matrix structure is

Table 2: Effect of methionine supplementation in TCFY extender on enzyme leakage during cryopreservation of Jamnapari bucks semen

Stages	Additive concentration (mM)	AST ($u\,l^{-1}$)	ALT ($u\,l^{-1}$)	ACP ($u\,l^{-1}$)	AKP, ($u\,l^{-1}$)
	Control ©	69.30 ± 1.50^{d}	37.08 ± 0.79^{d}	28.10 ± 0.60^{d}	51.65 ± 0.83^{d}
	2.0 mM (T_1)	67.10 ± 1.30^{c}	35.02 ± 0.81^{c}	25.90 ± 0.62^{c}	49.46 ± 0.75^{c}
Post-diluted	2.5 mM (T_2)	65.40 ± 1.11^{a}	32.79 ± 0.80^{a}	24.15 ± 0.51^{a}	47.46 ± 0.55^{a}
	5.0 mM (T_3)	66.38 ± 1.46^{b}	33.69 ± 0.83^{b}	25.06 ± 0.62^{b}	48.46 ± 0.95^{b}
	Control ©	239.6 ± 1.28^{D}	157.2 ± 0.84^{D}	68.23 ± 0.65^{D}	171.6 ± 0.81^{D}
	2.0 mM (T_1)	207.2 ± 1.25^{C}	124.7 ± 0.82^{C}	51.94 ± 0.61^{C}	155.5 ± 0.79^{C}
Post- thaw	2.5 mM (T_2)	193.2 ± 1.24^{A}	110.8 ± 0.78^{A}	44.81 ± 0.64^{A}	148.5 ± 0.82^{A}
	5.0 mM (T_3)	199.3 ± 1.26^{B}	116.6 ± 0.79^{B}	47.85 ± 0.60^{B}	151.5 ± 0.84^{B}

AST-ALT= Asparate and Alanine amino-transferases, ACP-AKP= Acid and Alkaline phosphatase, $u\,l^{-1}$=Unit per liter. Mean ± SE (n=48) values bearing different superscripts within the stage (a, b, c / A, B, C) in a column differ significantly ($P<0.05$).

destroyed (Sikka, 2004). However, the antioxidant capacity of sperm cells is insufficient in preventing oxidative stress during the freeze-thawing process (Storey, 1997). Al-Ahmad *et al.* (2008) demonstrated that the inclusion of amino acids in certain farm animal extenders can serve as antioxidants, safeguarding sperm from the detrimental impact of ROS and enhancing post-thaw sperm motility, viability and fertility. Methionine is one of the essential amino acids that contain sulfur and is essential for protein synthesis. Due to its role as a glutathione precursor, a tripeptide that combats reactive oxygen species and shields cells from oxidative stress, it possesses antioxidant properties. Furthermore, methionine plays a crucial role in the synthesis of polyamines, which are involved in nucleus and cell division processes. It is also a key provider of methyl groups for DNA and other molecules during methylation reactions (Bouyeh, 2012).

Moreover, Reed and Rrenius (1977) demonstrated that sulfur compounds, such as methionine, can bind with lead and eliminate it from tissues, thus playing a crucial role in detoxification. In addition, El-Battawy and El-Nattat (2018) elucidated that methionine supplementation (1.5, 2.5 and 5.0 mM) improves per cent sperm motility and declined non-viable sperm, and acrosomal abnormalities in post-thawed buck semen. In addition, Toker and Dogann (2023) observed significant improvement in post-thawed seminal attributes in Kivircik ram ejaculate supplemented with methionine (2.5 and 5 mM), cysteine (1 and 2 mM), and butylated hydroxytoluene (1 and 2 mM). Present observations align with the recent findings of Verma *et al.* (2023), who found that treatment with 2.5 mM methionine had a significant positive impact on various aspects of sperm quality in Murrah bull's semen after thawing. These improvements included increased progressive motility, enhanced livability, improved plasma membrane integrity, greater sperm penetration distance, reduced sperm abnormalities and decreased seminal plasma enzyme leakage.

In the present study, post-thaw sperm motility was significantly ($p<0.05$) and strongly correlated with post-thaw per cent livability (r= 0.93, 0.98, 0.96 & 0.99), per cent acrosomal integrity (r= 0.97, 0.98, 0.96 & 0.99), per cent plasma membrane activity (r= 0.97, 0.82, 0.88 & 0.98) and with sperm penetration distance (r= 0.93, 0.96, 0.94 & 0.97) however, it was negatively correlated with per cent sperm abnormality (r= -0.64, -0.70, -0.60 & -0.58), seminal plasma AST (r= -0.96, -0.99, -0.96 & -0.99), seminal plasma ALT (r= -0.95, -0.98, -0.96 & -0.99), seminal plasma ACP (-0.96, -098, -0.96 & -0.99) and seminal plasma AKP (r= -0.94, -0.97, -0.95 & -0.98), respectively, in control and methionine treated group (T_1, T_2, T_3). These findings on correlation coefficients between control and methionine-treated groups observed in Jamnapari buck semen synchronize with the observations of Verma (2023) in Murrah bull semen.

In conclusion, the use of methionine in a tris-citrate-fructose-yolk extender can improve the sperm quality and fertility potential of post-thawed spermatozoa of Jamnapari buck. Moreover, the addition of methionine to cryopreserved semen resulted in significant improvements ($p<0.05$) in seminal attributes (motility, livability, acrosomal integrity, sperm plasma membrane integrity and sperm penetration distance) and significantly ($p<0.05$) lower sperm abnormalities and enzyme leakage. Therefore, It is recommended to use methionine (2.5 mM) for the freezing of buck semen to get an objective result.

Acknowledgment

The authors thank the Dean, C.V. Sc. and A.H., ANDUAT Ayodhya for providing the necessary facility for experiment.

Authors' contribution: S. Srivastava, B. Singh, R.R. Singh: Conceptualized and designed the experiment; **J. Singh and K. Alam:** Carried out the statistical analysis, interpretation of data and figure; **K. Alam, P. Kumar and H.C. Verma:** Drafted the manuscript; **R. Kumar, S.K. Verma and A.K. Verma:** Technical assistance; **R.K.Verma and Saurabh:** Carried out the biochemical analysis; **R. Kumar and A. Alam:** Revising, editing and proofreading of manuscript; **A. Jaiswal and N. Singh:** Semen collection and management of buck during whole experimental period.

Funding: Partial financial support was received from RKVY, Gov. of India.

Research content: The research content of the manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that they have no conflict of interest.

Data availability: All the data analyzed for this study are included. Any extra data of interest if needed are available from the Corresponding author upon reasonable request.

Consent to publish: All the authors have read the draft and agree to publish the manuscript in its present form in the *Journal of Environmental Biology.*

References

Abdulkareem, T.A., F.F. Ibrahim, M.S. Hassan, O.A. Mohamed and W.E. Lateef: Effect of adding amino acids combinations to tris extender for improving post cryopreserved semen characteristics of Holstein bulls. *Biochem. Cell. Arch.,* **20**, 697-701 (2020).

Al Ahmad, M.Z., G. Chatagnon, L.A. Briand, M. Moussa, D. Tainturier, M. Anton and F. Fieni: Use of glutamine and low density lipoproteins isolated from egg yolk to improve buck semen freezing. *Reprod. Domest. Anim.,* **43**, 429-436 (2008).

Andrabi, S.M.H.: Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Domest. Anim.,* **44**, 552-569 (2009).

Bouyeh, M.: Effect of excess lysine and methionine on immune system and performance of broilers. *Ann. Biol. Res.,* **3**, 3218-3224 (2012).

Bucak, M.N., P.B. Tuncer, S. Sarıozkan, N. Başpınar, M. Taspınar, K. Çoyan and D. Oztuna: Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology,* **61**, 248-253 (2010a).

Çoyan, K., N. Başpınar, M.N. Bucak, P.P. Akalın, M.B. Ataman, A.D. Omur and S. Sarıozkan: Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. *Res. Vet. Sci.,* **89**, 426-431 (2010).

Dewry, R.K., B.C. Deka, D. Bhuyan, R.K. Biswas, S. Sinha, Z. Hussain and S.B. Hazarika: Effect of vitamin E on the quality of frozen buck semen. *Indian J. Small Rumin.,* **21**, 343-346 (2015).

El-Battawy, K.A. and W.S. El-Nattat: Cryopreservation of buck semen with emphasis on its chilling and its characteristics after some amino acids supplementation. *Curr. Sci. Int.,* **7**, 731-736 (2018).

Fayyaz, M.H., M. Ahmad and N. Ahmad: Survival of buffalo bull spermatozoa: effect on structure and function due to alpha-lipoic acid and cholesterol-loaded cyclodextrin. *Andrologia,* **49**, e12652 (2017).

Fernandez-Santos, M.R., F. Martinez-Pastor, V. García-Macías, M.C. Esteso, A.J. Soler, P.D. Paz and J. J. Garde: Sperm characteristics and DNA integrity of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. *J. Androl.,* **28**, 294-305 (2007).

Forouzanfar, M., S.F. Ershad, S.M. Hosseini, M. Hajian, S. Ostad-Hosseini, A. Abid, M. Tavalaee, A. Shahverdi, A.V. Dizaji and M.H.N. Esfahani: Can permeable super oxide dismutase mimetic agents improve the quality of frozen–thawed ram semen?. *Cryobiology,* **66**, 126-130 (2013).

Gangwar, C., S.D. Kharche, R. Ranjan, S. Kumar, A.K. Goel, S.K. Jindal and S.K. Agarwal: Effect of vitamin C supplementation on freezability of Barbari buck semen. *Small Rumin. Res.,* **129**, 104-107 (2015).

Ghadimi, M., S.D. Sharifi, A. Najafi and H. Mohammadi: Gallic acid supplementation partially ameliorates reproductive aging in rooster breeders by improving semen quality, sperm kinetics, hormones, and antioxidant status. *Poultry Sci.,* **103**, 103842 (2024).

Griveau, J.F., E. Dumont, P. Renard, J.P. Callegari and D. Le Lannou: Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *Reproduction,* **103**, 17-26 (1995).

Gundogan, M.: Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi rams. *Turk. J. Vet. Anim. Sci.,* **30**, 95-100 (2006).

Hancock, J.L.: A staining technique for the study of temperature-shock in semen. *Nature,* **167**, 323-324 (1951).

Irvine, D.S.: Glutathione as a treatment for male infertility. *Rev. Reprod.,* **1**, 6-12 (1996).

Levine, R.L., B.S. Berlett, J. Moskovitz, L. Mosoni and E.R. Stadtman: Methionine residues may protect proteins from critical oxidative damage. *Mech. Ageing Dev.,* **107**, 323-332 (1999).

Lorton, S.P., H.L. Kummerfeld and R.H. Foote: Polyacrylamide as a substitute for cervical mucus in sperm migration tests. *Fertil. Steril.,* **35**, 222-225 (1981).

Makarevich, A.V., E. Kubovicova, A.V. Sirotkin and J. Pivko: Demonstration of the effect of epidermal growth factor on ram sperm parameters using two fluorescent assays. *Vet. Med.,* **55**, 581-589 (2010).

Michael, A.J., C. Alexopoulos, E.A. Pontiki, D.J. Hadjipavlou-Litina, P. Saratsis, H.N. Ververidis and C.M. Boscos: Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim. Reprod. Sci.,* **112**, 119-135 (2009).

National Research Council (NRC). Nutrient requirement of small ruminant (sheep, goat, cervids and new world camelids). The National academies press, Washington, D. C., 384 pages (2007).

Oehninger, S., M. Morshedi, S.L. Weng, S. Taylor, H. Duran and S. Beebe: Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. *Reprod. Biomed. Online,* **7**, 469-476 (2003).

Patra, R.C., D. Swarup and S.K. Dwivedi: Antioxidant effects of α tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. *Toxicology,* **162**, 81-88 (2001).

Perumal, P., S. Selvaraju, S. Selvakumar, A.K. Barik, D.N. Mohanty, S. Das, R.K. Das and P.C. Mishra: Effect of pre-freeze addition of cysteine hydrochloride and reduced glutathione in semen of crossbred Jersey bulls on sperm parameters and conception rates. *Reprod. Domest. Anim.,* **46**, 636-641 (2011).

Ranjan, R., A.K. Goel, N. Ramachandran, S.D. Kharche and S.K. Jindal: Effect of egg yolk levels and equilibration periods on freezability of Jamunapari buck semen. *Indian J. Small Rumin.,* **21**, 32-36 (2015).

Ranjan, R., A.K. Goel, S.D. Kharche, N. Ramachandran, C. Gangwar and S.K. Jindal: Comparison of normal and dual staining techniques for evaluating viability and acrosome status in frozen thawed buck spermatozoa. *Indian J. Small Rumin.,* **20**, 50-53 (2014).

Ranjan, R., N. Ramachandran, S.K. Jindal, N.K. Sinha, A.K. Goel, S.D. Kharche and A.K.S. Sikarwar: Effect of egg yolk levels on keeping quality of Marwari buck semen at refrigeration temperature. *Indian J. Anim. Sci.,* **79**, 662-664 (2009).

Ranjan, R., R. Priyadharsini, A. K. Goel, B. Singh, S. Kumar, S.D. Kharche and S.K. Jindal: Effect of membrane stabilizer on the freezability of buck semen. *Indian J. Anim. Sci.,* **87**, 435-436 (2017).

Rao, T.K.S., N. Kumar, N.B. Patel, I. Chauhan and S. Chaurasia: Sperm selection techniques and antioxidant fortification in low grade semen of bulls. *Vet. World,* **6**, 579-585 (2013).

Reed, D.J., and S. Orrenius: The role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochem. Biophys. Res. Commun.,* **77**, 1257-1264 (1977).

Reed, D.J.: Glutathione: toxicological implications. *Annu. Rev. Pharmacol. Toxicol.,* **30**, 603-631(1990).

Sariozkan, S., P.B. Tuncer, M.N. Bucak, S. Buyukleblebici, H. Kinet and A. Bilgen: The effects of different egg yolk concentrations used with soy bean lecithin-based extender on semen quality to freeze bull semen. *Eurasian J. Vet. Sci.,* **26**, 45-49 (2010).

Shah, S.A.H., S.M.H. Andrabi and I.Z. Qureshi: Freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of curcumin (diferuoyl methane) in semen extender. *Andrologia,* **49**, 12713 (2017).

Sikka, S.C.: Andrology lab corner: role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J. Androl.,* **25**, 5-18 (2004).

Storey, B.T.: Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol. Hum. Reprod.,* **3**, 203-213 (1997).

Taylor, C.T.: Antioxidants and reactive oxygen species in human fertility. *Environ. Toxicol. Pharmacol.,* **10**, 189-198 (2001).

Toker, M.B. and I. Dogan: Methionine, cysteine and butylated hydroxytoluene enhance cryosurvival of ram semen on post-thaw and post-incubation time points. *Trop. Anim. Health Prod.,* **55**, 218 (2023).

Tuncer, P.B., M.N. Bucak, S. Sarıozkan, F. Sakin, D. Yeni, İ.H. Çigerci, and O. Buyukleblebici: The effect of raffinose and methionine on frozen/thawed Angora buck (*Capra hircus ancryrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology,* **61**, 89-93 (2010).

Turner, R.M.O. and S.M. McDonnell: Alkaline phosphatase in stallion semen: characterization and clinical applications. *Theriogenology,* **60**, 1-10 (2003).

Verma, A.K., R. Kumar, S. Srivastava, K. Alam, R. Kumar, D.S. Sakhahari and S.K. Verma: Methionine as a semen additive to improve Murrah buffalo (*Bubalus bubalis*) semen cryopreservation. *Indian J. Vet. Sci. Biotechnol.,* **19**, 39-43 (2023).

Waisum, O.W., H. Chen and P.H. Chow: Male genital tract antioxidant enzymes-their ability to preserve sperm DNA integrity. *Mol. Cell Endocrinol.,* **250**, 80–83 (2006).

Watson, P.F.: The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.,* **60**, 481-492 (2000).

Watson, P.F.: Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Vet. Rec.,* **97**, 12-15 (1975).

On Line Copy