RESEARCH ARTICLE

Methionine as a Semen Additive to Improve Murrah Buffalo *(Bubalus bubalis)* Semen Cryopreservation

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Abstract

The aim of the present investigation was to evaluate the impact of methionine at different concentrations (2.0 mM and 2.5 mM) on the seminal parameters before and after cryopreservation of extended Murrah bull semen. Thirty-six ejaculates, collected with the aid of an artificial vagina twice a week from six Murrah bulls, were included in the study. Each ejaculate was diluted in a Tris-citric-acid-fructose-egg-yolk-glycerol (TFYG) extender keeping 80 million sperm per mL and split into three equal aliquots, which were supplemented with methionine {0.0, 2.0 and 2.5 mM; Treatment C, T1 and T2, respectively), filled in 0.25 mL straws, cooled to and equilibrated for 4 h at 4° C, and then frozen in LN2 vapour. Frozen straws were thawed at 37°C for 30 seconds in a water bath for the post-thaw evaluation. Sperm motility, percent live sperm, sperm abnormalities, *in-vitro* fertility tests (HOST and CMPT), and enzyme leakage (AST, ALT) were evaluated at both post-dilution and post-thaw stages. The results revealed that the addition of methionine, at both 2.0 mM and 2.5 mM concentrations significantly ($P<0.05$) improved sperm motility, sperm livability, and HOS-positive spermatozoa. Further, the sperm abnormalities and enzyme leakage were significantly ($P<0.05$) lower in methionine-treated groups than in control group. The results were the best with 2.5 mM methionine supplementation. In conclusion, the study showed that methionine at a concentration of 2.5 mM exhibited superior protection of sperm structures and functions as compared to 2.0 mM methionine and the control group.

Key words: Cryopreservation, *In vitro* fertility tests, Methionine, Murrah, Semen.

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Introduction

Murrah, the world's well-known breed of buffalo, is commonly used for grading up local or non-descript buffalo germplasm in India (Ahirwar *et al.,* 2018). There is enough probability for genetic improvement of livestock by the implementation of various reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET), and *in-vitro* embryo production. Selective breeding to improve the species requires an artificial insemination (AI) program using semen from males with high genetic merit, as it is a more successful, economical, and simple technique. However, the process of cryopreservation exerts physiological, osmotic, and chemical stresses on the sperm membrane and sperm structure, which may result in damage to post-thawed spermatozoa (Ozkavukcu *et al.,* 2008). Poor keeping quality and freezability of buffalo bull semen are recorded by many research workers (Chaudhari *et al.,* 2015). Buffalo spermatozoa are highly susceptible to osmotic changes occurred during the process of freezing and thawing and are more susceptible to oxidative stress damage for high content of polyunsaturated fatty acids than cattle (Khan *et al.,* 2021). Addition of antioxidants in semen extenders reduce the ROS mediated chemical damage to spermatozoa during cryopreservation and thawing by neutralizing free radicals (Bilodeau *et al.,* 2001). Usually, the semen additives may maintain frozen-thawed seminal quality and enhance the conception rate (Perumal *et al.,* 2011).

Methionine acts as a precursor amino acid for glutathione. Moreover, its sulphur content has shown to chelate lead and remove it from tissues, and thereby plays a vital role in detoxification and it is proven as a protective when added to bull semen extender (Bucak *et al.,* 2010). Moreover, El-Battawy and El-Nattat (2018) stated that methionine significantly improved post-thaw sperm motility percentage and reduced acrosomal defects and dead sperm percentage.

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It is noteworthy that methionine assumes a crucial function in the antioxidant defense mechanism through its propensity to readily react with oxidants, resulting in the formation of methionine sulfoxide (Kim *et al.,* 2014). This study was aimed to evaluate the impact of varying doses of methionine on motility, morphology, *in-vitro* fertility tests (HOST & CMPT), and seminal plasma enzyme leakage in cryopreserved Murrah bull semen.

Materials and Methods

The additive methionine (USB, Corporation Cleveland, OH, USA) and other chemicals used in this study were obtained from Merck, KGaA 64271 Darmstadt, Germany.

Animals and Semen Collection

This study utilized semen samples obtained from six mature Murrah bulls with superior genetic merit and fertility capacity. The Murrah bulls were raised at the Deep Frozen Semen Laboratory of the College of Veterinary Science and Animal Husbandry, ANDUAT, Kumarganj, Ayodhya in Uttar Pradesh, India. They were kept under standard nutritional and managerial conditions. A total of thirty-six ejaculates were collected twice a week from six Murrah bulls using an artificial vagina during the period of January to March 2023. Following collection, the ejaculates were promptly placed in a warm water bath set at a temperature of 34°C until evaluated within 20 minutes.

Semen Extension, Freezing and Thawing

The ejaculates with visual motility exceeding 75 % were diluted using a Tris-citric-acid-fructose-egg-yolk-glycerol (TFYG) extender with a pH of 7.0. Diluted semen were divided into three equal aliquots, and methionine was added later @ 0, 2, and 2.5 mM to the control C and treatment groups T1 and T2, respectively. Diluted semen samples were filled into 0.25 mL French straws, sealed by the ISEVO filling, sealing, and printing machine (IMV, France), and equilibrated at 4°C for 4 hours. After equilibration, the straws were frozen in liquid nitrogen vapour, 4 cm above the liquid nitrogen, for 10 minutes and plunged into the liquid nitrogen for storage. After being stored for 24 hours, the frozen straws were thawed at 37°C for 30 seconds in a water bath for microscopic evaluation.

Evaluation of Semen

Semen samples were evaluated for pre-freeze (on dilution) and post-thaw sperm motility, viability and morphology using the established protocols described by Hafez and Hafez (2000). The hypo-osmotic swelling test was conducted following the protocol outlined by Jayendran *et al.* (1984). The cervical mucus penetration test was conducted and evaluated using the grading system developed by Hollinshead *et al.* (2003). The seminal plasma was separated from freshly diluted and frozen-thawed samples using centrifugation at 3000 rpm for 20 minutes. The levels of aspartate and alanine aminotransferases (AST and ALT) were determined using commercially available kits on the BEACONB Auto^{200} serum analyzer.

Statistical Analysis

The data were presented as the mean and standard error of the mean (SEM), and analyzed using one-way analysis of variance (ANOVA) with Graph Pad Prism 5 software.

Results and Discussion

The effects of methionine on the sperm parameters of diluted and frozen bovine semen are presented in Table 1. Semen samples cryopreserved with 2.5 mM methionine in TFYG extender had significantly ($p<0.05$) higher percent pre-freeze and post-thaw sperm motility, viability and morphological normal sperm followed by 2.0 mM methionine as compared to the control extender ($P < 0.05$). The mean post-thaw motility recorded was in agreement with the observation of Toker and Dogan (2023), higher than that reported by Bucak *et al.* (2010), and lower than the finding of El-Battawy and El-Nattat (2018). Our findings on sperm viability were in tune with Alam *et al.* (2023), and lower than the findings of El-Sheshtawy and El-Nattat (2020), but higher than those reported by Dheerib *et al.* (2020).

During cryopreservation, more than 50% of spermatozoa are usually injured due to the formation of ice crystals in the extracellular and intracellular environments and increasing solute concentration (Watson, 1995). In addition, cryopreservation also induces sublethal damage to the spermatozoa, resulting in loss of motility, viability, *in-vivo* fertilizing potential, with deterioration of sperm, acrosomal and plasma membrane integrity, and damage to deoxyribonucleic acid (Bucak *et al.,* 2010). Moreover, peroxidation of the membranes of mammalian spermatozoa damages the structure of the lipid matrix due to the attacks of ROS, which are produced through univalent reduction of oxygen during cryopreservation (Sikka, 2004). The ROS produced during the freezing and thawing of sperm can overwhelm the natural antioxidant defense system and damage the spermatozoon membrane, resulting in poor viability and low fertilization potential (Bailey *et al.* 2001). Methionine is a thiol-containing amino acid that penetrates the cell membrane easily, enhancing intracellular glutathione biosynthesis *in-vivo* (Patra *et al.,* 2001) and improves sperm cryoprotection. In addition, methionine residues have been provided to scavenge ROS and thus are important antioxidants. Moreover, Mohammed *et al.* (2014) observed that supplementation of methionine and glutathione to bull semen extenders improved post-cryopreservation semen characteristics due to their dual roles as antioxidant and cryoprotectant agents. Abdulkareem *et al.* (2020) noticed that the methionine-fortified groups had higher initial motility, live sperm count, and acrosomal integrity ($P \leq 0.05$) than the control group.

Table 1: Effect of methionine (2.0 mM & 2.5 mM) supplementation in Tris extender on sperm quality parameters and enzyme leakage during cryopreservation of Murah bulls semen

Stages	Additive concentration (mM)	Progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	HOS reactive sperm (%)	SPD (mm/h)	AST (µmole/L)	ALT (µmole/L)
Post-diluted	Control (T_0)	72.75 ± 0.27^{a}	78.69 ± 0.39^{a}	6.83 ± 0.25^{b}	47.25 ± 0.31^{a}	32.81 ± 0.34^{a}	92.47 ± 0.82^{b}	22.19 ± 0.31^{b}
	2.0 mM (T_1)	74.08 ± 0.28^{b}	78.81 ± 0.46^{ab}	6.36 ± 0.24^{ab}	48.28 ± 0.34^{b}	32.92 ± 0.34^{a}	91.03 ± 0.88^{ab}	21.75 ± 0.28^{ab}
	2.5 mM (T_2)	74.83 ± 0.28^{c}	79.36 ± 0.41^{b}	6.14 ± 0.24^{a}	49.03 ± 0.32^{c}	34.06 ± 0.27^{b}	90.44 ± 0.73^{a}	21.58 ± 0.30^{a}
Post- thaw	Control (T_0)	46.0 ± 0.73^{A}	63.67 ± 0.67^{A}	17.33 ± 0.71^{A}	35.0 ± 0.89^{A}	25.67 ± 0.7^{A}	157.5 ± 3.10^{A}	32.0 ± 0.73^{A}
	2.0 mM (T_1)	53.0 ± 0.73^{B}	66.17 ± 0.79^{B}	14.33 ± 0.71^{B}	38.5 ± 0.62^{B}	28.0 ± 0.73^{B}	147.3 ± 3.07^{B}	29.0 ± 0.73^{B}
	2.5 mM (T_2)	56.0 ± 0.73^{C}	69.17 ± 0.75^{C}	13.33 ± 0.71^{C}	39.5 ± 0.62^{C}	30.33 ± 0.88^{C}	127.2 ± 3.06^{C}	27.17 ± 0.60^{C}

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

The average percent HOS-reactive spermatozoa were significantly (p<0.05) higher in extender containing 2.5 mM methionine at both pre-freeze and post-thaw stage followed by 2.0 mM methionine as compared to the control extender (P < 0.05, Table 1). Our findings tuned with the observation of Alam *et al.* (2023), but were lower than those reported by Chaudhary *et al.,* (2015) and higher than those observed by Arboud *et al.* (2020). Plasma membrane functionality and integrity are critical sperm parameters for evaluating sperm quality because an intact plasma membrane is an essential borderline for sperm cell survival (Makarevich *et al.,* 2010). It also plays critical roles in sperm metabolism, capacitation, the acrosome reaction, and finally sperm-oocyte fusion (Forouzanfar *et al.,* 2013).

In the present study, mean SPD in bovine cervical mucus of post-diluted and post-thawed semen was observed to be significantly (P<0.05) higher in extender containing 2.5 mM methionine, followed by 2.0 mM methionine and the control extender (P < 0.05, Table 1). Our findings were higher than those reported by Kumar (2014). However, most *in-vitro* studies for determining sperm penetration distance do not show sufficient determination of bull fertility (Gillan *et al.,* 2008). In contrary, Bhatia *et al.* (2007) reported that pregnancy rates were highest in bulls with the greatest sperm penetration distance in bovine cervical mucus. Methionine serves as a precursor amino acid for glutathione and contributes to the preservation of membrane functionality and sperm motility. Glutathione is a tripeptide thiol compound with various crucial roles in cellular physiology. These roles encompass safeguarding the cell against oxidative stress, facilitating protein and DNA synthesis, and facilitating gamete fertilization (Schafer and Holzmann, 2000).

The average values of AST and ALT activities in plasma of post-thawed semen were found to be significantly (P<0.05) higher in extender containing 2.5 mM methionine, followed by 2.0 mM methionine and the control extender (P < 0.01). At post-dilution stage the values of 2.0 mM methionine were however statistically similar with 2.5 mM methionine and even control extender (Table 1). Seminal plasma is a complex mixture of secretions from various accessory sex glands, enriched with factors that modulate the fertilizing ability of sperm (Turner and McDonnell, 2003). The enzyme AST-ALT activities in seminal plasma are good indicators of sperm quality because they represent the sperm plasma membrane stability, and higher enzyme activity indicates enzyme leakage due to compromised membrane stability (Gundogan, 2006). Moreover, AST is essential for the metabolic processes of spermatozoa by providing energy for their survival, motility, and fertility (Perumal *et al.,* 2011). Methionine is one of two proteinogenic (sulphur containing) essential amino acids that contain sulfur. Because it is a glutathione precursor, a tripeptide that reduces ROS and thus protects cells from oxidative stress, for its antioxidant properties (Bouyeh, 2012).

Conclusions

The findings of current study seem to validate the positive impact of antioxidant-methonine supplementation in Tris-citric-acid-fructose-egg-yolk-glycerol (TFYG) extender on the parameters of Murrah bull spermatozoa. Supplementation of extender with 2.5 mM methionine significantly enhanced quality of post-thawed Murrah bull semen when compared with 2.0 mM methionine and both were superior over the control extender.

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