RESEARCH ARTICLE

Caffeine Supplementation in Semen Extender Protects Buffalo Spermatozoa from Cryodamage

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Abstract

Caffeine is widely known for its phosphodiesterase enzymes inhibiting, and its powerful reactive oxygen species scavenging property. This study aimed to evaluate the effect of caffeine supplementation @ 0 mM, 1 mM, 3 mM and 5 mM in AndroMed® extender on cryopreservation of Jaffarabadi buffalo semen. Twenty-four semen ejaculates (6/bull) with >70% initial sperm motility were obtained from four mature Jaffarabadi bulls using AV. The ejaculates were split-diluted in an egg-yolk-free AndroMed® extender supplemented with different concentrations of caffeine and were cryopreserved using a standard protocol. The semen samples were evaluated for sperm quality parameters as well as oxidative stress parameters, viz., lipid peroxidation, and Glutathione-S-transferase (GST) enzyme activity in seminal plasma at pre-freeze (after equilibration) and post-thaw stage. The levels of caffeine had a significant effect on all these parameters, except sperm abnormalities, at both pre-freeze and post-thaw stages. Supplementation of caffeine in semen extender at 1 mM and 3 mM concentration showed a significant ($p<0.05$) increase in post-thawed sperm motility (62.04 ± 0.84, 61.25 ± 1.01%), viability (64.21 ± 0.88, 64.25 ± 0.84%), acrosome integrity (58.08 ± 1.08, 57.30 ± 0.93%) and plasma membrane integrity (52.75 ± 0.89, 52.71 ± 0.74%) and a significant ($p<0.05$) decrease in oxidative damage as evident by lower lipid peroxidation (MDA 7.62 ± 0.41, 7.80 ± 0.70 µM) and GST enzyme activity (31.15 ± 1.36, 29.54 ± 0.54 nmol CDNB/mL/min) as compared to control and 5 mM caffeine. The study concludes that the post-thaw quality of frozen semen of Jaffarabadi buffalo bulls improves significantly with decreased oxidative stress if the AndroMed® extender is supplemented with 1 and 3 mM concentration of caffeine over control.

Keywords: Caffeine, Cryodamage, Jaffarabadi buffalo, Oxidative markers, Semen cryopreservation.

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Introduction

Buffalo farming has made remarkable progress mainly because of controlled breeding and genetic up-gradation through the application of AI using cryopreserved semen which has become the most viable biotechnological tool for faster genetic dissemination in many species. Although cryopreservation is considered to be an integral part of AI technique, it is often associated with inflicting considerable damage to spermatozoa of buffalo bulls than cattle by affecting the sperm motility, viability, plasma membrane, acrosomal and DNA integrity due to the unique physiology of the buffalo spermatozoa and higher polyunsaturated phospholipids levels in the plasma membrane (Andrabi, 2009). It was also observed that the cryopreservation process induced apoptosis in cryopreserved buffalo bull spermatozoa along with a reduction in semen quality parameters (Khan *et al.,* 2009).

During cryopreservation, lipid peroxidation of the bio-membrane takes place, which increases the level of reactive oxygen species (ROS), causing oxidative stress and reducing the post-thaw semen quality due to loss of membrane fluidity and integrity, failing sperm oocyte fusion (Colagar *et al.,* 2013). Motility, plasmalemma integrity, and fertility of bull semen are negatively correlated with lipid peroxidation levels (Kasimanickam *et al.,* 2007). Therefore, a balance between ROS production and antioxidant is necessary for sperm stability. Glutathione-S-transferases (GSTs) are postulated as important detoxifying enzymes that catalyze reduced glutathione-dependent reactions in cellular protection against oxidative stress and toxic chemicals (Hayes *et al.,* 2005). In response to ROS production, the GSTs can alter the levels of cellular GSH. There are three major families of GSTs,

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viz., cytosolic, mitochondrial, and microsomal involved in regulating different metabolic processes, signaling, and stress responses (Kunieda *et al.,* 2005).

Over the years, several antioxidant additives have been used to increase post-thawed sperm quality and fertility. One such additive is caffeine which is a heterocyclic compound (1, 3, 7-tri-methylxanthine) with antioxidant and antiradical properties (Yashin *et al.,* 2013), promoting hyper-activation of sperm in bulls (Marquez and Suarez, 2004). Caffeine, a potent antioxidant found in coffee beans, is widely known to enhance sperm fertility by inhibiting phosphodiesterase enzyme leading to deposition of sperm intracellular cAMP, which plays an important role in sperm motility (Buffone *et al*., 2014). It also possesses characteristic powerful scavenging property, which protects the sperm from membrane damage and DNA fragmentation during freeze-thawing (Esterbauer and Cheeseman, 1990). The present study was planned to evaluate the effect of supplementing Caffeine in AndroMed® extender on cryopreservation of buffalo bull semen.

Materials and Methods

Semen Collection

The study was undertaken on semen of four sexually mature and healthy Jaffarabadi buffalo bulls, aged between 4 to 6 years, maintained under identical nutritional and managerial conditions at Cattle Breeding Farm, Junagadh Agricultural University, Junagadh (India). The bulls were fed as per the Minimum Standard Protocol of the Government of India (2000), with concentrate, dry fodder, and green fodder @ 0.8 kg, 1.6 kg, and 4.16 kg/100 kg body weight, respectively, and the mineral mixture was supplemented @ 60 gm/day. They had free access to clean drinking water throughout the day. As a routine, bathing and grooming were carried out daily, and exercise was practiced in a rotatory bull exerciser a day before semen collection. Semen was collected in the morning hours twice a week from the bulls using a sterile artificial vagina (IMV, Danish model). Immediately after collection, a tube containing the ejaculate was kept in a water bath at 34°C and assessed for various seminal attributes adopting standard procedures (Tomar, 1984). Soon after evaluation, the freshly collected semen samples were diluted @ 80 million sperm/mL with extender AndroMed® (Minitube, Germany).

Extender Additive and Dilution

Caffeine (HiMedia Lab. Pvt. Ltd, Mumbai) stock solution 0.1 M was prepared and stored in a refrigerator. At the time of use, it was added in the extended semen in a way to get 1 mM, 3 mM, and 5 mM of final concentration. Egg-yolk-free concentrated AndroMed® was used as a semen extender containing phospholipids, Tris (2-Amino-2-hydroxymethyl-propane-1, 3-diol), citric acid, sugars, antioxidants, buffers, glycerol and purest water, including antibiotics according to the EC Directive 88/407 (Tylosin, Gentamicin, Spectinomycin, Lincomycin). One part of AndroMed® was diluted with four parts of distilled water just before use and was kept in a water bath for equilibrium at 34°C before semen collection.

The selected semen ejaculates with >70% initial sperm motility were diluted with the final volume of the extender as per dilution rate (80 million sperm/mL) depicted by a Bovine Accucell Photometer, IMV) and were divided into four equal aliquots. Subsequently, Aliquot-1 was kept as control without any additive (0 mM caffeine), while Aliquots - 2, 3, and 4 were supplemented with caffeine @ 1 mM, 3 mM and 5 mM, respectively.

Processing and Evaluation of Semen

After final dilution and incorporation of different concentrations of caffeine, the semen aliquots were filled and sealed in medium French straws (0.5 mL, 15 straws per aliquot) by an automatic filling and sealing machine (MRS1 Dual, IVM, France). Straws were then transferred to a cold handling cabinet (Macro Scientific Pvt. Ltd, New Delhi) maintained at 4°C for 4 h of equilibration.

After equilibration, the content of six-seven straws from each aliquot was collected and evaluated for pre-freeze sperm motility, viability, abnormality (eosin-nigrosin stain), and plasma membrane integrity (HOST, 150 mOsm) and acrosomal integrity (Giemsa stain). Seminal plasma was also collected from semen of all four aliquots by centrifugation of samples at 700 g for 10 min and stored in cryovial at -20°C for assay of lipid peroxidation and Glutathione-S-Transferases (GSTs) activity. The remaining straws were vapor frozen using the conventional method and submerged in liquid nitrogen at -196°C. The cryopreserved straws were thawed at 37 °C for 30 s in a water bath and were again evaluated for post-thaw sperm motility, viability, abnormality, plasma membrane integrity (HOST), and acrosomal integrity adopting standard procedures, and the seminal plasma samples collected from frozen-thawed straws were stored at -20°C till further analysis.

Seminal Plasma Biochemical Assays

The stored seminal plasma samples were thawed and analyzed for lipid peroxidation and Glutathione-S-Transferase (GSTs) activity. The peroxidative membrane damage was determined in terms of malondialdehyde (MDA) produced using the standard procedure and kit provided by Sigma Aldrich (Saint Louis, USA). Glutathione-S-Transferases (GSTs) were estimated by using kits procured from HiMedia Lab. Pvt. Ltd., Mumbai, adopting 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as per the instructions of the manufacturer of assay kit (Hayes *et al.,* 2005).

Statistical Analysis

The data obtained for various parameters were expressed as Mean ± SEs for pre-freeze and post-thawed semen. The data were analyzed by one-way ANOVA and Duncan's multiple

range test using SPSS software version 20.0 to determine significant differences between caffeine levels at p<0.5.

Results and Discussion

The findings on the effect of adding caffeine in semen diluent at different concentrations (0 mM, 1 mM, 3 mM and 5 mM) on sperm motility, viability, morphology, osmotic resistance test, acrosomal integrity, and oxidative stress parameters at pre-freeze (post-equilibration) and post-thaw stages of cryopreservation of Jaffarabadi buffalo semen are presented in Tables 1-3.

Sperm Motility, Viability, and Morphology

The mean percent sperm motility and viability at pre-freeze and post-thaw stages in extender supplemented with 1 mM and 3 mM Caffeine were significantly (p<0.01) higher than the control extender and 5 mM Caffeine group. The post-thaw sperm motility in 5 mM caffeine supplemented extender was also found significantly (p<0.01) higher over control (Table 1). The mean sperm abnormality was, however, non-significantly lower at pre-freeze in the control group and post-thaw stage in 1 mM caffeine group as compared to that of 5 mM caffeine and control group. These observations concurred with the report of Shukla and Misra (2014) on Murrah buffalo semen with the same concentrations of caffeine. Similarly, a significant (p<0.05) increase in the sperm motility and viability with decreased sperm abnormalities of cattle or buffalo spermatozoa using caffeine as an additive at different concentrations has been reported by different workers ,viz., (Singh and Raina, (2000) at 4 mM; Zaiden et al. (2006) at 2 mM and 4 mM, and Barakat et al. (2015) at 5 mM caffeine.

The significantly improved sperm motility and viability with reduced sperm abnormality on supplementation of caffeine in ram (Jenagrad *et al.,* 2018; Hamid and Ibrahim, 2019; Rateb *et al.,* 2020) and camel semen (El-Bahrawy, 2017) have also been reported. Caffeine, when added to semen, would markedly stimulate sperm motility through activation of cAMP production, which can lead to a decrease in apoptotic and dead/necrotic sperm numbers. The cAMP stimulates sperm motility by direct action on the axoneme of the tail or by indirectly acting on the cell membrane as a secondary messenger (Garbers and Kopf, 1980). The effect of caffeine on sperm motility may be species-specific and dose-dependent (Spalekova *et al.* (2014). Caffeine has a stimulatory effect on respiration, and it is reported to inhibit certain

Table 1: Sperm motility, viability and abnormality in Jaffarabadi buffalo bull semen with different concentration of caffeine at pre-freeze and post-thaw stage of cryopreservation (Mean ± SE)

	Sperm motility (%)		*Sperm viability (%)*		*Sperm abnormality (%)*	
Groups/ Caffeine conc	*Pre- freeze*	*Post-thaw*	*Pre- freeze*	*Post-thaw*	*Pre- freeze*	*Post-thaw*
Control 0 mM	73.96 ± 1.12^{a}	53.54 ± 0.82^{a}	76.08 ± 0.96^{a}	56.67 ± 0.70^{a}	7.04 ± 0.27	10.42 ± 0.37
Caffeine 1 mM	80.42 ± 1.04^{b}	62.04 ± 0.84^{c}	82.54 ± 0.94^{b}	64.21 ± 0.88^{c}	7.17 ± 0.45	10.25 ± 0.37
Caffeine 3 mM	80.21 ± 0.97^{b}	61.25 ± 1.01^{c}	82.50 ± 0.87^{b}	64.25 ± 0.84^{c}	7.67 ± 0.49	10.75 ± 0.49
Caffeine 5 mM	75.62 ± 0.97^{a}	57.08 ± 0.79^{b}	78.50 ± 0.84^{a}	59.71 ± 0.76^{b}	8.50 ± 0.45	11.25 ± 0.36

Means with different superscripts within column differ significantly at (p<0.01) level.

Table 2: Acrosome integrity and HOS reacted sperm in Jaffarabadi buffalo bull semen with different concentration of caffeine at the pre-freeze and post-thaw stage of cryopreservation (Mean ± SE)

	Acrosome integrity (%)		*HOS reacted sperm (%)*	
Groups/ Caffeine conc	*Pre- freeze*	*Post-thaw*	*Pre- freeze*	*Post-thaw*
Control 0 mM	71.17 ± 0.90^{a}	51.08 ± 0.72^{a}	65.62 ± 0.93^{a}	45.54 ± 0.70^{a}
Caffeine 1 mM	76.83 ± 0.98^{b}	58.08 ± 1.08^{c}	71.75 ± 0.99^{b}	52.75 ± 0.89^{c}
Caffeine 3 mM	77.25 ± 1.03^{b}	57.30 ± 0.93^{bc}	71.79 ± 0.99^{b}	52.71 ± 0.74^{c}
Caffeine 5 mM	72.96 ± 0.93^{a}	54.00 ± 0.82^{ab}	67.79 ± 0.95^{a}	49.29 ± 0.76^{b}

Means with different superscripts within column differ significantly at p<0.05 level.

Table 3: Lipid peroxidation and Glutathione-S-Transferases (GST) in Jaffarabadi bull semen with different concentrations of caffeine at pre-freeze and post-thaw stage of cryopreservation (Mean ± SE)

	Lipid peroxidation (μM)		*GST (nmol/ml/min)*	
Groups/ Caffeine conc	*Pre- freeze*	*Post-thaw*	*Pre- freeze*	*Post-thaw*
Control 0 mM	7.69 ± 0.57	11.02 ± 1.23^{b}	31.46 ± 3.14	38.91 ± 2.03^{b}
Caffeine 1 mM	7.15 ± 0.58	7.62 ± 0.41^{a}	25.73 ± 2.51	31.15 ± 1.36^{a}
Caffeine 3 mM	6.71 ± 0.67	7.80 ± 0.70^{a}	25.31 ± 0.91	29.54 ± 0.54^{a}
Caffeine 5 mM	6.63 ± 0.50	7.34 ± 0.57^{a}	28.96 ± 1.45	33.49 ± 1.53^{a}

Means with different superscripts within column differ significantly at p<0.05 level.

enzymes in glycolysis. Furthermore, increased intracellular cAMP levels activate glycogen phosphorylase and break glycogen into simple sugars (Singh and Raina, 2000).

Acrosome and Plasma Membrane Integrity

The mean percent sperm acrosomal integrity and HOST reactive sperm in extender supplemented with 1 mM and 3 mM caffeine were significantly ($p<0.01$) higher at pre-freeze as well as at post-thaw stage as compared to those of 5 mM caffeine and control groups (Table 2). These findings concurred well with those of Singh and Raina (2000) and Shukla and Misra (2014) in buffalo bull semen, Srivastava and Kumar (2014) in HF bull semen, and Jenagrad *et al.* (2018) and Rateb *et al.* (2020) in ram semen for significant ($p<0.05$) increase in acrosome integrity and/or HOS reactivity with caffeine supplementation in the extender. Further, Pereira *et al.* (2000) in cattle bull and buck semen, Hamid and Ibrahim (2019) in ram semen, and El-Bahrawy (2017) in camel semen also showed a positive effect of caffeine on percent acrosome integrity.

For successful fertilization, a high proportion of sperm with an intact membrane is necessary (Spalekova *et al.,* 2014). During the first step of fertilization, spermatozoa secrete their acrosomal contents (the 'acrosome reaction') to penetrate the extracellular matrix of the oocyte and reach the oocyte plasma membrane at the site of fertilization (Gadella and Evans, 2011). Hence the presence of more sperm with intact acrosomes at the site of fertilization is beneficial in increasing the conception rate. Caffeine increased intracellular calcium and an immediate hyper-activation of sperm (Col'as *et al*., 2010).

Lipid Peroxidation and Glutathione-S-Transferases

The mean MDA concentrations in sperm-free seminal plasma at the pre-freeze stage were not significantly ($p>0.05$) different between levels of caffeine. However, at the post-thaw stage, all groups with Caffeine supplementation showed a protective effect and maintained MDA level, but in control, it increased significantly at the post-thaw stage (Table 3). The overall mean GST activities observed in the control group at a pre-freeze and post-thaw stage of Jaffarabadi buffalo semen were 31.46 ± 3.14 and 38.91 ± 2.03 nmol CDNB/mL/min, respectively. The mean GSTs activities were significantly ($p<0.05$) lower in all caffeine-added samples than in control, particularly at the post-thaw stage (Table 3). The findings suggested that adding caffeine has decreased spermatozoal damage, irrespective of its concentration. Similarly, Jenagrad *et al.* (2018) reported a significantly higher concentration of MDA in ram semen extended with 0 mM caffeine (4.1 nmol/mL) followed by 4 mM caffeine, 1 mM caffeine, 0.5 mM caffeine and the least in 2 mM caffeine (2.4 nmol/mL). Kumar *et al.* (2014) reported increased activity of GSTs in post-thawed semen than in fresh semen of buffalo bulls. There was a paucity of data regarding lipid peroxidation and GST activity in bull and buffalo semen after addition of caffeine as a semen additive. However, the researchers have noted beneficial effects of various other additives on lipid peroxidation of cattle and/or buffalo bull semen (Selvaraju *et al.,* 2009; Patel *et al.,* 2019; Ahmed *et al.,* 2021).

Cryopreservation adversely affects the sperm quality and functionality as well as an increase in oxidative stress, lipid peroxidation and apoptotic-like events. Sperm damage during cryopreservation causes cellular enzymes like GSTs to leak into seminal fluid. GSTs are 24 kDa enzymes, glycosylated proteins on the sperm acrosome and are required for sperm oocyte interaction during fertilization. GSTs are essential sperm antioxidant enzymes involved in cell protection against oxidative stress and toxic chemicals, preserving sperm function and fertilizing ability. MDA production is higher in frozen-thawed spermatozoa compared with that of fresh spermatozoa, suggesting the generation of ROS by several potential sources during cryopreservation, such as aromatic amino oxidase enzyme activity in dead sperm. Thus the increased number of dead sperm may be one of the attributing factors to increased levels of LPO.

Conclusion

From the study it is concluded that the supplementation of caffeine in semen extender at 1 mM and 3 mM concentration has significant ($p<0.05$) impact on sperm motility, viability, acrosome integrity and plasma membrane integrity with decreased oxidative damage as evident by lower lipid peroxidation and GST enzyme activity as compared to extender containing 0 mM (control) or 5 mM caffeine. Thus, the post-thaw quality of frozen semen of Jaffarabadi buffalo bulls can be improved significantly if the AndroMed® dilutor is supplemented with 1 and 3 mM concentration of caffeine. However, actual fertility trials are warranted to conclude the ideal concentration of caffeine to be used as an additive.

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