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Caffeine in Tris Egg Yolk Citrate Extender Improves the Antioxidant Potential of Cryopreserved Surti Buck Semen

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ABSTRACT

The study was conducted to evaluate the antioxidant action of caffeine at various concentrations in TEYC extenders. In the present study, a total 64 ejaculates (16/buck) were collected from all four Surti bucks (age>12 months) i.e. two collections/week for 8 weeks. Ejaculates at each collection were pooled and divided into four groups *viz*. C1 (Control), C2, C3 and C4 having caffeine concentrations @ 0, 1, 3 and 5mM in TEYC extender, respectively with $100x10^6$ sperm/ml. Oxidative stress marker for lipid peroxidation (Malondialdehyde, MDA) and antioxidant (Glutathione, GSH) were determined at initial, pre-freeze and post-thaw; whereas, motility degeneration rate (MDR) of sperms was evaluated at pre-freeze and post-thaw. A decrease in GSH and an increase in MDA levels with elapse of time were found to be significant ($p<0.01$) between different phases. The MDA levels were observed to be significantly ($p<0.01$) different between groups that were highest in C1 and lowest in C4 in contrast to GSH levels that were highest in C4 and lowest in C1 groups. MDR was significantly ($p<0.01$) higher at post-thaw than pre-freeze in all the groups. Moreover, MDR was significantly ($p<0.01$) lower in C2 group as compared with other groups at both the pre-freeze and post-thaw stage. It was therefore concluded that for cryopreservation of Surti buck semen, supplementation of caffeine in TEYC extender has dose-dependent increase in antioxidant defense and decrease in oxidative stress as indicated by higher GSH and lower lipid peroxidation, respectively but 1mM caffeine concentration in TEYC is optimum and better owing to the lowest MDR.

Key words: Antioxidant, Caffeine, Cryopreserved Semen, Glutathione, Malondialdehyde, Motility Degeneration Rate, Surti Buck

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INTRODUCTION

India has about 148.88 million goats the are excellent sources of meat, milk, fiber and skin (20th Livestock Census, 2019). Due to the surge in demand for various products it is vital to maximize reproduction efficiency which invariably depends on successful conception. Artificial insemination (AI) is crucial for genetic gain among various assisted reproductive technologies. For successful AI, it is of prima facie importance that semen quality is preserved especially during cryopreservation. Unfortunately, during cryopreservation sperm get exposed to several oxidative stress instances. Even though cryoprotectants in the extenders reduce the detrimental effects of freezing, sperm membrane is still vulnerable to reactive oxygen species that may decrease sperm quality, especially by lipid peroxidation (Lenzi *et al*., 2002; Bucak *et al*., 2007). Lipid peroxidation that leads to malondialdehyde (MDA) production can be used to assess the extent of oxidative stress and damage in semen. This oxidative stress is generally combated by endogenous antioxidants i.e. reduced glutathione. Reduced glutathione indicates the antioxidant status in the semen. Rao *et al.* (2012) have reported significant increase in lipid peroxidation (MDA levels) and enzyme leakage in extra cellular medium of crossbred HF bulls' semen following freezing and thawing without addition of antioxidants implying the necessity of adding antioxidants in semen. Sinha *et al.* (2004) have reported that during freezing and thawing of frozen buck sperms presence of glutathione has protective action that significantly decreased the leakage and release of key enzymes like hyaluronidase, alkaline phosphatase, alanine transaminase, aspartate transaminase and lactate dehydrogenase from the sperm.

Thus increased MDA and decrease in reduced glutathione indicate oxidative stress and vice versa. To augment endogenous antioxidant defense, antioxidants can be added to extenders. One such option caffeine (1,3,7-Trimethylxanthine), that belongs to methylxanthine group with antioxidant and antiradical characteristics (Yashin *et al*., 2013). As sperm motility is one of the foremost criteria of functional evaluation of sperm (Eo *et al*., 2019) motility degeneration rate (MDR) seems to be an accurate indicator of good quality of preserved semen. Singh *et al*. (2011) has demonstrated that caffeine significantly enhanced *in vitro* capacitation of bovine spermatozoa and increased sperm penetration distance in 2% polyacrylamide gel during capacitation implying positive impact of caffeine supplementation in the medium possibly due to hyperactive spermatozoa. Considering these aspects along with south Gujarat being the native tract of the Surti breed of goats, the present study was planned to evaluate the antioxidant potential of caffeine in tris egg yolk citrate extender in cryopreserved semen of Surti bucks.

MATERIALS AND METHODS

Location of study, selection of bucks and their management: The present study was conducted between December, 2022 and April, 2023. at the Dept of Vety Gynaecology & Obstetrics, College of Vety Sci & Animal Husbandry, Kamdhenu University, Navsari. Navsari is a coastal city located in the south Gujarat region with a location as 20°57' to 20°95' North latitude and 72°56' to 72°93' East longitude at an elevation of 9 meters above the mean sea level. Four apparently healthy Surti bucks (>12 months old) were selected from the AICRP, Goat (Livestock Farm Complex, LFC), Kamdhenu University, Navsari. The selected bucks were already dewormed and vaccinated against common diseases *viz*. PPR and FMD.

Collection of semen and group formation: The bucks were trained for 45 days to ejaculate semen in an artificial vagina using a dummy doe. Semen was collected twice per week from each buck for 8 weeks i.e., total of 64 semen ejaculates from all four bucks. Semen was collected using separate artificial vaginas (AV) of 8-inch for different bucks with optimum inner temperature (40°C-42°C) and pressure having a pinch of lubricating jelly (K-Y by Johnson and Johnson, France) at the opening. A graduated glass tube was attached with the latex cone at the other end of the AV. The tube and AV were covered appropriately to prevent thermal shock to the semen ejaculates. All plastic ware, glass wares and rubber items used were sterile and collection was done aseptically.

After every collection, freshly ejaculated semen was assessed for colour, pH, volume, density, mass activity, sperm concentration and total sperm count. Density was scored as 1 (Milky/Watery), 2 (Thin creamy), 3 (Creamy) or 4 (Thick creamy) by observing tube with ejaculate with bright light in background. Scoring for mass activity was done by observing a drop of ejaculate under microscope (low power) and based on observing waves and eddies were graded from scale 1 to 5 (ranging from '+' to '+++++'). All four ejaculates collected at a time from four bucks were pooled and extended with egg yolk citrate extender to make final concentration of $100x10^6$ sperm/ml. 10% egg yolk in Tris-citric acid-fructose buffer (Tris-2.42g, Citric acid-1.34g, Fructose-1.00 g, Streptomycin-0.10 g, Penicillin-1 lakh IU and Milli Q Water-80 ml at pH-6.8 -7.0) extender that was made by mixing and centrifugation at 3000 rpm for 4 minutes.

The diluted samples of semen having ≥70 % motility were considered for further processing and were divided into four aliquots containing different concentration of caffeine (Loba Chemical Pvt. Ltd, India) *viz*. C1 (Control), C2, C3 and C4 had caffeine concentrations @ 0, 1, 3 and 5mM, respectively in TEYC extender and cryopreserved using standard procedure in which after equilibration of semen straws at 4°C for about 4 hrs, the floating rack holding the straws was placed in a manual vapour freezing unit (Minitube, Germany) for 10 minutes wherein straws remained 5 centimeters above liquid nitrogen in vapor phase and after completion of freezing, the straws were directly and quickly plunged into liquid nitrogen container

Assessment of parameters for antioxidant potential: Oxidative stress parameter i.e. malondialdehyde (MDA) for lipid peroxidation and reduced Glutathione (GSH) as antioxidant parameter were assessed at initial (before equilibration at 4°C for about 4 hours), pre-freeze (before cryopreservation) and post-thaw (after 24 hrs. of cryopreservation), Meanwhile, the Motility Degeneration Rate (MDR) was assessed pre-freeze and post-thaw.

Lipid peroxidation (i.e. MDA production) was assessed by the method described by Banday *et al*. (2017). Reduced glutathione was estimated by precipitating diluted semen sample with trichloroacetic acid, centrifuging at 1000xg for 5 min, mixing supernatant with Tris–EDTA buffer and 5,5 - dithio-bis-2-nitrobenzoic acid (DTNB) followed by keeping at room temperature for 5 min and measuring absorbance of sample and standard solution by spectrophotometer at 412 nm against the reagent blank. Calculations were done as GSH = (OD of Test/ OD of Standard)x Concentration of matching standard. Motility Degeneration Rate (MDR) was determined by calculating the percent decrease in motility at pre-freeze and post thaw as compared to the initial stage using the formula and the motility was examined at initial, pre-freeze and post thaw stages. At the end of each time the motility degeneration rates were determined by the following formula.

$$\text{MDR at various hrs} = \frac{\text{Motility (0 hrs) - Motility at various hrs}}{\text{Motility (0 hrs)}} \times 100$$

(Campos *et al*., 2004)

Statistical analysis

Descriptive statistical analysis was carried out and mean±SEM was calculated for MDA, reduced GSH and MDR for all the groups. Comparisons between means were made based on one way ANOVA using Duncan New Multiple Range Test as post-hoc test for mean separation at 5% and 1% levels of significance.

RESULTS AND DISCUSSION

Lipid Peroxidation: The initial mean MDA (nmol/10^8 sperm) level differed non-significantly (p>0.05) between all the groups (Table 1). While, pre-freeze and post-thaw mean MDA levels were observed lower in C4 group (5mM caffeine) when compared to other concentrations of C1 (0mM caffeine), C2 (1mM) and C3 (3mM) of caffeine-treated groups. Further, the mean MDA level of different groups was significantly (p<0.01) higher at the post-thaw as compared to initial and pre-freeze stage.

The lower post-thaw mean MDA level in Surti buck semen was observed with 5mM (C4) followed by 3mM (C3) and 1mM (C2) caffeine concentration in TEYC extender. MDA production at different caffeine concentrations in present study could not be comparatively reviewed adequately due to lack of similar studies. However, a study by Jenagrad *et al*. (2018) in Ghezel ram semen with soybean lecithin extender while refrigeration and cryopreservation showed non-significant (p>0.05) decrease as well as lowest post-thaw MDA concentration at 2mM as compared to 0.5, 1 and 4mM caffeine concentrations in various dilutors.

Table 1: Effect of different concentrations of caffeine on MDA level of cryopreserved Surti buck semen

Groups	MDA (nmol/10^8 sperm) (n=16)			F value	P value
	Initial	Pre-freeze	Post-thaw		
Control	5.25^{a}_{y}±0.11	7.17^{a}_{x}±0.13	12.26^{a}_{w}±0.28	382.61**	0.00
1mM Caffeine	5.15^{a}_{y}±0.19	6.92^{a}_{x}±0.12	09.81^{b}_{w}±0.19	231.61**	0.00
3mM Caffeine	5.00^{a}_{y}±0.16	6.57^{b}_{x}±0.10	08.55^{c}_{w}±0.23	103.41**	0.00
5mMCaffeine	4.91^{a}_{y}±0.15	6.16^{c}_{x}±0.12	07.07^{d}_{w}±0.15	59.90**	0.00
F value	1.001	14.71**	103.47**	--	--
P value	0.40	0.00	0.00	--	--

$^{a-d}$Mean values bearing different superscripts within columns differ significantly at p<0.01.

$_{w-z}$Mean values bearing different subscripts between columns differ significantly at p<0.01.(**p<0.01)

On the contrary, the mean concentrations of MDA (nM/ml) level were not affected in 0.1mM, 0.2mM, 0.3mM and 0.4mM groups compared with control group at 48 hr of chilled preservation (4°C) as reported by Abd El-Hamid (2019) in Barki ram.

Mammalian sperm membranes containing unsaturated fatty acids are vulnerable to LPO in the presence of ROS, decreasing sperm quality. The antioxidant system comprising reduced GSH has been described as a defense functioning mechanism against the lipid peroxidation of semen and is important in maintaining sperm motility and viability.

Reduced Glutathione (GSH): The mean GSH level (nmol/ml) differed significantly ($p<0.05$ and $p<0.01$) at initial, pre-freeze and post-thaw stage between all the groups (Table 2). The mean GSH level was significantly higher ($p<0.01$) in C4 (5mM Caffeine) as compared with other groups C1 (0mM), C2 (1mM) and C3 (3mM) and significant ($p<0.01$) decreasing trend pattern was observed between different time phases.

The higher post-thaw mean GSH level in Surti buck semen was observed with 5mM (C4) followed by 3mM (C3) and 1mM (C2) caffeine added groups. However, literature directly associated with the effect of caffeine supplementation on GSH levels in semen were not available, the present results suggest the intricate antioxidant mechanism of caffeine. GSH as a hydrophilic antioxidant, protects cells against reactive oxygen (ROS) and nitrogen (RNS) species (Rauhala *et al*., 2005; Jozefczak *et al*., 2012). Therefore, during oxidative stress and excess ROS generation, GSH levels may be depleted. Various studies have postulated the role of caffeine as a reactive oxygen scavenger (ROS) that especially tackles $OH^{\cdot}$ (hydroxyl radical). Moreover, the major beneficial role of caffeine is attributed to its highly effective antioxidant metabolites *i.e.,* 1-methylxanthine as well as methyluric acid as observed by Lee (2000). Furthermore, caffeine has been reported to prevent Fenton's reaction-induced GSH oxidation. Therefore, adding caffeine to the medium containing GSH will lead to sparing effect on GSH causing unutilized GSH to accumulate and increase its levels. This could justify the results of

Table 2: Effect of different concentrations of caffeine on Reduced Glutathione (GSH, nmol/ml) level of cryopreserved Surti buck semen

Groups	GSH (nmol/ml) (n=16)			F value	P value
	Initial	Pre-freeze	Post-thaw		
Control	$5.70^{b}_{w}\pm0.17$	$4.64^{c}_{x}\pm0.17$	$3.26^{d}_{y}\pm0.12$	61.32**	0.00
1mM Caffeine	$5.83^{ab}_{w}\pm0.17$	$4.66^{c}_{x}\pm0.15$	$3.90^{c}_{y}\pm0.15$	38.32**	0.00
3mM Caffeine	$5.95^{ab}_{w}\pm0.17$	$5.22^{b}_{x}\pm0.16$	$4.37^{b}_{y}\pm0.17$	22.66**	0.00
5mMCaffeine	$6.22^{a}_{w}\pm0.15$	$5.71^{a}_{w}\pm0.16$	$4.82^{a}_{x}\pm0.14$	18.40**	0.00
F value	1.78*	10.32**	20.76**	--	--
P value	0.02	0.00	0.00	--	--

$^{a-d}$Mean values bearing different superscripts within columns differ significantly at $p<0.05$ &$p<0.01$.(* $p<0.05$ &**$p<0.01$)

$_{w-z}$Mean values bearing different subscripts between columns differ significantly at $p<0.01$.

Table 3: Effect of different concentrations of caffeine on Motility Degeneration Rate (MDR %) of cryopreserved Surti buck semen

Groups	MDR (%) (n=16)		F value	P value
	Pre-freeze	Post-thaw		
Control	$35.73^{a}_{x}\pm1.17$	$62.77^{a}_{w}\pm1.48$	206.11**	0.00
1mM Caffeine	$23.46^{b}_{x}\pm1.25$	$34.09^{d}_{w}\pm1.79$	23.75**	0.00
3mM Caffeine	$24.01^{b}_{x}\pm2.08$	$46.61^{c}_{w}\pm2.28$	53.56**	0.00
5mMCaffeine	$32.25^{a}_{x}\pm1.10$	$54.94^{b}_{w}\pm1.95$	66.39**	0.00
F value	13.24**	41.86**	--	--
P value	0.00	0.00	--	--

$^{a-d}$Mean values bearing different superscripts within columns differ significantly at $p<0.01$.

$_{w-z}$Mean values bearing different subscripts between columns differ significantly at $p<0.01$.(**$p<0.01$)

higher GSH levels in supplemented groups as compared to control--caffeine-supplemented groups.

Motility Degeneration Rate: The mean MDR (%) at pre-freeze and post- thaw were observed significantly ($p<0.01$) lower in C2 group (1mM caffeine) when compared to other concentrations C1 (0mM), C3 (3mM) and C4 (5mM) of caffeine treated groups and significant increasing trend was observed in mean MDR between pre-freeze and post-thaw in different groups (Table 3).

Significant ($p<0.05$) increase of MDR at 24 hr post-thaw as compared to pre-freeze in all the groups of present study was observed. This findings was similar to those reported at intervals of refrigerated temperature by Amarjeet *et al*. (2019) at 12 , 24, 36 and 48 hr; by Atara *et al*. (2019) at 30, 60 and 120 minutes and by Kumar *et al*. (2022) at 24 hr, 36 hr and 48 hours. Kant *et al*. (2020) showed increased sperm kinetics and motility in Marwari stallions in groups due to treatment with caffeine among which 2mM caffeine showed significant improvement in post-thaw sperm motility and other functional attributes. In crossbred bull semen, moderate and considerable improvement in frozen semen quality has also been reported by Srivastava and Kumar (2006) while comparing effects of some additives on freezability of semen.

CONCLUSIONS

During cryopreservation of Surti buck semen, supplementation of caffeine in semen extender has dose-dependent increase in antioxidant defense and decrease in oxidative stress Caffeine concentration @1mM in TEYC is optimum owing to the lowest MDR in semen samples. Future studies may focus on benefits of adding caffeine supplementation during semen cryopreservation in relation to conception. Moreover, the work can be replicated for species other than goats.

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CONFLICT OF INTEREST

The authors don't have any conflict of interest in the conduct of this experiment.

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