RESEARCH ARTICLE

Effect of Adding Caffeine in Tris Extender on Critical Quality Parameters of Cryopreserved Surti Buck Semen

Darshita V. Bhut[1]*, Chandubhai T. Khasatiya[1], Virendra Kumar Singh[2]

Abstract

The study was conducted to evaluate the effect of adding caffeine in Tris egg yolk citrate glycerol (TEYCG) extender on critical quality parameters of cryopreserved Surti buck semen. Four mature Surti bucks (aged >1 year) were selected. Total 64 ejaculates were collected @ 2 ejaculates per week/buck over eight weeks period. At every collection, ejaculates of all 4 bucks were pooled and divided into four equal aliquots, diluted with TEYCG extender (having 100 x10^6 sperms/mL), added with caffeine as 0 mM (T1, control), 1 mM (T2), 3 mM (T3) and 5 mM (T4) in the four aliquots, respectively, and were cryopreserved and stored for 24 h in LN_2. Individual sperm motility, viability, morphology and functional membrane integrity of sperms were evaluated at initial, pre-freeze (after equilibration for about 4 h at 4°C) and post-thaw stages. Individual motility, viability, morphologically normal and HOST-reacted sperms decreased significantly ($p<0.01$) with successive phases of storage. At initial, pre-freeze and post-thaw stage, normal and HOST reacted sperms as well as at pre-freeze and post-thaw stage individual motility and viability were significantly ($p<0.01$) higher in T2 followed by T3 group than the other groups. It was thus concluded that caffeine supplementation in TEYCG extender has beneficial effect on sperm quality parameters particularly at 1 mM followed by 3 mM as compared to higher (5 mM) caffeine concentration or non-added control.

Key words: Caffeine, Cryopreservation, Semen quality, Surti buck.

Ind J Vet Sci and Biotech (2024): 10.48165/ijvsbt.20.4.18

Introduction

Goats are commonly referred as "poor man's cow" (MacHugh and Bradley, 2001). With goat population of 148.88 million (20th Livestock Census, 2019), its husbandry is integral to livestock industry in India. Its contribution in terms of milk, meat and fibre production has huge socio-economic impact. Due to surge in demand for various goat products, it is vital to maximize reproduction efficiency that invariably depends on successful conception. Since, artificial insemination is indispensable for improving genetic gain, successful semen cryopreservation is critical for maintaining semen quality using cryoprotectant and antioxidants. Cryoprotectant reduces detrimental effects of freezing but still sperm membrane is vulnerable to reactive oxygen species that may decrease sperm quality especially by lipid peroxidation (Lenzi *et al*., 2002; Bucak *et al*., 2007). Endogenous antioxidant defense can be augmented using exogenous antioxidants such as caffeine (1,3,7-Trimethylxanthine), which is a member of methylxanthine group with antioxidant and antiradical characteristics (Yashin *et al*., 2013). Considering Surti goats native to south Gujarat, and the need to preserve critical semen quality parameters while cryopreservation, this study was planned to evaluate the effect of adding caffeine to tris extender on critical quality parameters of cryopreserved Surti buck semen.

Materials and Methods

The study was conducted at Department of Veterinary Gynaecology and Obstetrics, College of Veterinary science and Animal Husbandry, Kamdhenu University, Navsari (Gujarat, India). Navsari district is located in coastal region of south Gujarat having latitude and longitude at 20°57′ to 20°95′ North and 72°56′ to 72°93′ East, respectively, with an elevation of 9 meters above sea level. Four apparently healthy Surti bucks of age greater than one year were selected for the study from All India Coordinated Research Project (AICRP-Goat) at Livestock Farm Complex of Kamdhenu University, Navsari. The selected bucks were dewormed and vaccinated for Foot and Mouth Disease and Peste des Petits Ruminants.

[1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396450, Gujarat, India

[2]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396450, Gujarat, India

Corresponding Author: Darshita V. Bhut, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396450, Gujarat, India, e-mail: drdarshitabhut28@gmail.com.

How to cite this article: Bhut, D. V., Khasatiya, C. T., & Singh, V. K. (2024). Effect of Adding Caffeine in Tris Extender on Critical Quality Parameters of Cryopreserved Surti Buck Semen. Ind J Vet Sci and Biotech. 20(4), 84-88.

Source of support: Nil

Conflict of interest: Authors don't have any conflict of interest.

Submitted 11/03/2024 **Accepted** 25/04/2024 **Published** 10/07/2024

The bucks after being selected for study were trained for 45 days using dummy does for ejaculating in artificial vaginas (AVs) of about 8-inch size and applied with lubricating K-Y jelly (Johnson & Johnson, France) at the front opening. Each buck ejaculated aseptically in different AVs that were jacketed along with tube to avoid thermal shock to sperms. AVs were maintained at optimum temperature (40-42°C) and pressure. A total of 64 semen ejaculates were collected at twice a week interval from all the four bucks over eight weeks period. Ejaculates were screened and only those having greater than 70% motility were further selected for pooling. The pooled semen of all 4 bucks was extended with tris egg yolk citrate glycerol extender for final concentration of sperm at 100 x 10^6/mL. Ingredients like Tris (2.42 g), Citric acid (1.34 g), Fructose (1.00 g), Streptomycin (0.10 g), Penicillin (1 lakh IU) were dissolved in 80 mL Milli-Q Water at pH of 6.8-7.0. The egg yolk (10 %) and glycerol (6%) were mixed thoroughly. Extended semen was divided into four equal aliquots and caffeine (Loba Chemical Pvt. Ltd, India) was added @ 0, 1, 3 and 5 mM in aliquot T1 (Control), T2, T3 and T4, respectively. The extended aliquots were filled and sealed in French straws and cryopreserved in LN_2 vapour after 4 h of equilibration at 4°C using standard protocol and stored for 24 h. At initial, pre-freeze (after equilibration) and post-thaw stages critical quality parameters of semen such as individual sperm motility, sperm viability, sperm morphology and functional membrane integrity (hypo-osmotic swelling test, HOST) were evaluated.

Descriptive statistical analysis was done to obtain mean ± SE of different parameters at different stages. One way ANOVA along with DNMRT as post-hoc test was conducted to compare means and observe differences at 5% level of significance (Snedecor and Cochran, 1994).

Results and Discussion

Results of individual sperm motility, sperm viability, sperm morphology and functional membrane integrity (at initial, pre-freeze and post-thaw) of Surti buck semen supplemented with different concentrations of caffeine to the TEYCG extender are presented in Table 1, 2, 3 and 4, respectively.

Individual Sperm Motility

Mean individual sperm motility percent differed non-significantly ($p>0.05$) at initial stage between groups, but after equilibration and post-thawing it was significantly higher ($p<0.01$) in T2 (1 mM Caffeine) as compared to other groups. A significant ($p<0.05$) decreasing trend was observed in sperm motility from initial to post-thaw stage (Table 1).

Significantly higher individual sperm motility was observed at pre-freeze and post-thaw stage with 1 mM (T2) caffeine followed by 3 mM (T3) over 5 mM (T4) and control extender (T1). Scientific literature was not adequately available to compare with similar concentration of caffeine in TEYC for buck semen. However as reported by Agarwal *et al.* (2010), 7 mM caffeine treated Sirohi buck semen showed progressive motility non-significantly ($p>0.05$) higher after dilution and equilibration, and significantly ($p<0.05$) higher after thawing compared to control group. Similarly, overall mean sperm motility differed non-significantly after equilibration and was significantly ($p<0.01$) higher at 16 h post-thaw in Beetal and Sirohi goat semen with tris extender supplemented with 2 mM caffeine (Goswami *et al.,* 2021). Caffeine supplementation has also shown significant beneficial effects on cryopreseved semen of different species of animals, *viz.,* ram spermatozoa with 4 mM of caffeine (Spalekova *et al.,* 2014), buffalo sperm at 1 mM and 4 mM caffeine in EYTG (Singh and Raina, 2000; Shukla and Misra, 2014), and 1 mM caffeine in AndroMed® (Chavda *et al.*, 2022), and in HF and HF×Hariana bull semen at 0.54 mM caffeine in tris egg yolk extender (Srivastava and Kumar, 2014). Beneficial effects either dose-dependent or species-specific, can be explained by the fact that caffeine is a cyclic phosphodiesterase inhibitor that markedly increase and maintain respiration and motility of ejaculated bovine spermatozoa (Garbers *et al.,* 1971). Inhibition of phosphodiesterase increases intracellular adenosine monophosphate leading to increased sperm motility.

Sperm Viability

Mean live sperm percent differed non-significantly ($p>0.05$) at initial stage between the groups, while after equilibration and post-thaw stage, the mean values were significantly ($p<0.01$) higher in T2 group (1 mM caffeine) followed by T3 (3 mM caffeine) as compared to other groups with a significant decreasing pattern from initial, pre-freeze to post-thaw stage

Table 1: Effect of different concentration of caffeine on individual sperm motility of cryopreserved Surti buck semen (n=16, Mean ± SE)

Groups (caffeine concentration)	Individual sperm motility (%)			F value	P value
	Initial	Pre-freeze	Post-thaw		
T1 (0 mM**)**	80.63^{a}_{w}±1.76	51.88^{b}_{x}±1.64	30.00^{d}_{y}±1.29	259.67**	0.00
T2 (1 mM**)**	82.81^{a}_{w}±1.29	63.44^{a}_{x}±1.56	54.69^{a}_{y}±1.91	80.29**	0.00
T3 (3 mM**)**	81.56^{a}_{w}±1.27	62.19^{a}_{x}±2.37	43.75^{b}_{y}±2.30	85.54**	0.00
T4 (5 mM**)**	81.25^{a}_{w}±1.40	55.31^{b}_{x}±2.35	36.88^{c}_{y}±2.09	125.90**	0.00
F value	0.41	7.52**	29.62**	--	--
P value	0.75	0.00	0.00	--	--

Mean values with different superscript (a-d) within a column and those with subscript (w-z) within the row differ significantly at $p<0.01$.

in all groups (Table 2). Variation in percent dead sperms showed trend opposite to that of live sperm percent at pre-freeze and post-thaw phase in different groups, and it being a fraction of 100, data is not depicted here.

Significantly ($p<0.01$) higher mean live sperm percent was observed with 1 mM (T2) followed by 3 mM (T3) caffeine as compared to 5 mM (T4) and control (T1) groups at pre-freeze and post-thaw stages in Surti buck semen. The trend of live sperm was same immediately on dilution but the differences were non-significant between groups. In other studies, mean live spermatozoa was significantly ($p<0.05$) higher after dilution, equilibration and post-thaw stage with 7 mM caffeine in Sirohi buck semen (Agarwal *et al.,* 2010) and with 2 mM caffeine in Beetal and Sirohi goat semen only at post-thaw stage (Goswami *et al.*, 2021). Significantly ($p<0.05$) higher post-thaw sperm viability percent with 1 mM caffeine in EYTG compared to 3 mM, 5 mM caffeine and control group in Murrah buffalo bulls (Shukla and Misra, 2014) and with 1 mM and 3 mM caffeine in Andromed® extender in Jaffarabadi bull semen (Chavda *et al*., 2022) has also been reported.

Other similar findings include significantly ($p<0.01$) improved post-thaw sperm viability in 1 mM caffeine concentration in soybean lecithin extender for Ghezel ram semen (Jenegrad *et al.,* 2018), with 4 mM caffeine in tris buffer egg yolk extender in buffalo bull semen (Singh and Raina, 2000) and with 0.54 mM caffeine in tris egg yolk extender in Holstein Friesian (HF) and HF×Hariana bull semen (Srivastava and Kumar, 2014). Cryopreservation of spermatozoa alters the structure and functions of cell membrane due to lipid peroxidation. This alteration decreases sperm viability as well as fertility and eventually results in sperm death. Caffeine increases intracellular cAMP level that may decrease number of apoptotic and dead/necrotic sperms.

Sperm Morphology

Mean normal/abnormal sperm percent differed significantly ($p<0.01$) between groups at all intervals of cryopreservation process. Abnormal sperm percent was significantly ($p<0.01$) lower in 1 mM (T2) group than all other groups at all three stages, and also increased significantly from the initial to post-thaw stage in all the treatment groups (Table 3). Reverse trend was observed in mean normal sperm percent in different groups, being a fraction of 100.

The results could not be compared due to lack of relevant studies of Surti buck semen. However, there are reports of decrease in percentage of morphological abnormalities and significantly ($p<0.01$) lower percentage of post-thaw sperm abnormality with 2 mM followed by 1 mM, 0.5 mM and 4 mM caffeine concentration in soybean lecithin extender as compared to control group in Ghezal ram semen (Jenagrad *et al*., 2018); decreased morphologically abnormal sperm percent in 0.1 mM than in 0.2 mM, 0.3 mM

Table 2: Effect of different concentrations of caffeine on live sperm (%) of cryopreserved Surti buck semen (n=16, Mean ± SE)

Groups (caffeine concentration)	Live sperm (%)			F value	P value
	Initial	Pre-freeze	Post-thaw		
T1 (0 mM)	83.06^{a}_{w}±1.64	53.94^{c}_{x}±1.71	35.56^{c}_{y}±1.27	237.69**	0.00
T2 (1 mM)	86.00^{a}_{w}±1.11	68.06^{a}_{x}±1.40	50.69^{a}_{y}±0.96	74.49**	0.00
T3 (3 mM)	83.56^{a}_{w}±1.08	65.69^{ab}_{x}±2.02	48.25^{b}_{y}±2.46	68.21**	0.00
T4 (5 mM)	83.31^{a}_{w}±1.67	60.38^{b}_{x}±2.62	40.13^{c}_{y}±1.77	105.12**	0.00
F value	0.94	9.95**	16.93**	--	--
P value	0.43	0.00	0.00	--	--

Mean values with different superscript (a-d) within a column and those with subscript (w-z) within the row differ significantly at $p<0.01$.

Table 3: Effect of concentrations of caffeine on abnormal sperm (%) of cryopreserved Surti buck semen (n=16, Mean ± SE)

Groups (caffeine concentration)	Abnormal sperm (%)			F value	P value
	Initial	Pre-freeze	Post-thaw		
T1 (0 mM)	9.56^{a}_{y}±0.47	13.63^{a}_{x}±0.79	17.38^{a}_{w}±0.75	32.58**	0.00
T2 (1 mM)	4.31^{c}_{y}±0.31	8.88^{c}_{x}±0.40	13.19^{b}_{w}±0.56	103.05**	0.00
T3 (3 mM)	6.50^{b}_{y}±0.37	9.69^{c}_{x}±0.37	14.50^{b}_{w}±0.65	69.75**	0.00
T4 (5 mM)	7.13^{b}_{x}±0.38	11.31^{b}_{w}±0.58	15.00^{b}_{w}±0.73	53.06**	0.00
F value	31.73**	13.93**	6.64*	--	--
P value	0.00	0.00	0.01	--	--

Mean values with different superscript (a-d) within a column and those with subscript (w-z) within the row differ significantly at $p<0.01$.

Table 4: Effect of different concentrations caffeine on HOST reacted spermatozoa (%) of cryopreserved Surti buck semen (n=16, Mean ± SE)

Groups (caffeine concentration)	HOST reacted sperm (%)			F value	P value
	Initial	Pre-freeze	Post-thaw		
T1 (0 mM**)**	78.06^{b}_{w}±0.96	66.94^{b}_{x}±1.20	56.75^{a}_{y}±1.24	87.32**	0.00
T2 (1 mM**)**	81.38^{a}_{w}±0.81	71.13^{a}_{x}±1.14	60.19^{a}_{y}±1.33	90.02**	0.00
T3 (3 mM**)**	79.31^{ab}_{w}±1.02	68.44^{ab}_{x}±1.07	58.00^{a}_{y}±1.26	90.32**	0.00
T4 (5 mM**)**	78.19^{b}_{w}±0.99	67.00^{b}_{x}±0.94	57.13^{a}_{y}±1.13	106.87**	0.00
F value	2.62*	3.22*	1.54	--	--
P value	0.05	0.03	0.21	--	--

Mean values with different superscript (a-d) within a column and those with subscript (w-z) within the row differ significantly at p<0.01.

and 0.4 mM caffeine concentration added in the tris-citric egg yolk extender group and control group in Barki ram (Abd El-Hamid, 2019), with 1 mM than with 3 mM or 5 mM caffeine in tris egg yolk glycerol extender at post-freeze stage in Murrah breed (Shukla and Misra, 2014) and in AndroMed® extender in Jaffarabadi breed (Chavda *et al*., 2022). Primary abnormalities occur during spermatogenesis that can't be changed in the ejaculated semen. Secondary abnormalities occurring during handling of the semen, agitation and examination of different parameters due to degenerative changes in the semen might be the cause of reduction observed in secondary abnormalities especially on bent tail and on free head.

Functional Plasma Membrane Integrity (%)

The mean HOST reacted sperm percent differed significantly between groups at different stages of cryopreservation process. HOST reacted sperm percentage was significantly (p<0.01) higher in 1 mM (T2) group as compared to other groups with a significant decreasing pattern from initial to post-thaw stage under all treatments (Table 4). Reverse trend was observed for mean HOST non-reacted sperm percent in different groups being fraction of 100.

The highest mean HOST reacted sperm percent observed with 1 mM as compared to other groups at various phases was in agreement with the significantly (p<0.01) higher values reported with 2 mM caffeine in tris extender in Beetal and Sirohi goat semen (Goswami *et al*., 2021) and 0.1 mM followed by 0.2 mM, 0.3 mM and 0.4 mM caffeine concentration and control group in Barki ram spermatozoa (El-Hamid, 2019), with 0.5 mM followed by 1 mM, 2 mM and 4 mM caffeine concentration in extender than control group in Ghezel ram semen (Jenagrad *et al*., 2018), with 0.54 mM caffeine in tris egg yolk extender as compared to control in HF and HF× Hariana bull semen (Srivastava and Kumar, 2014) and with 1 mM than 3 mM, 5 mM caffeine and control group in tris egg yolk glycerol extender in Murrah buffalo (Shukla and Misra, 2014). Further, Chavda *et al*. (2022) in their study on Jaffarabadi buffalo bull semen also showed significantly (p<0.05) higher mean HOST reacted sperm percent at pre-freeze and post-thaw stages in extender supplemented with 1 mM and 3 mM than 5 mM caffeine and control group.

Oxidative damages especially lipid peroxidation leads to sperm membrane damage. Caffeine as an antioxidant in extender may alleviate the oxidative stress and help in protecting the sperm membrane. This might be the reason of preservation of functional membrane integrity and higher percent of HOST-reacted sperms. Variable effect on motility, viability, morphology and functional plasma membrane integrity of the caffeine observed in the literature, might be due to species variation; preservation protocol; various extenders and concentration of caffeine used by various different research workers in different studies. Apparently, 1 mM seems to be the optimum caffeine concentration in TEYCG for maximum preservation of semen quality parameters during cryopreservation. In this study, it was observed that at higher caffeine concentrations, *i.e.* 3 mM and 5 mM the benefits were more than control, but less than 1 mM indicating some sort of detrimental effects of very high caffeine levels, the reasons need to be explored in future studies.

Conclusion

Caffeine supplementation in TEYCG extender has beneficial effect on motility, viability, morphology and functional plasma membrane integrity of buck sperm, which is best at 1 mM followed by 3 mM as compared to either control or 5 mM caffeine concentration.

Acknowledgements

Authors are thankful to Principal, Veterinary College and Project In-charge, AICRP on Goat (Livestock Farm Complex), Kamdhenu University, Navsari for providing necessary facilities and support to conduct this study.

Refrences

20th Livestock Census (2019). All India Report. Department of Animal Husbandry, Dairying and Fisheries, Govt of India, New Delhi.

Abd El-Hamid, I.S. (2019). Effect of adding different levels of caffeine in the extender on some biochemical constituents, enzymatic

activities and physical characteristics of chilled and frozen ram semen. *Reproduction in Domestic Animals, 54*(2), 225-233.

Agarwal, S., Saxena, A., & Sinha, N.K. (2010). Effects of semen additives in cryopreservation of Sirohi buck semen. *Indian Veterinary Journal, 87*(7), 667- 669.

Bucak, M.N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., & Akçay, A. (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology, 67*(5), 1060-1067.

Chavda, B.P., Vala, K.B., Solanki, G.B., Prajapati, S.G., & Singh, V.K. (2022). Caffeine supplementation in semen extender protects buffalo spermatozoa from cryodamage. *Indian Journal of Veterinary Sciences & Biotechnology, 18*(4), 1-5.

Garbers, D.L., First, N.L., Sullivan, J.J., & Lardy, H.A. (1971). Stimulation and maintenance of ejaculated bovine spermatozoan respiration and motility by caffeine. *Biology of Reproduction, 5*(3), 336-339.

Goswami, M.K., Sinha, S., Deka, B.C., Bhuyan, M., & Biswas, R.K. (2021). Effect of addition of vitamin E and caffeine on quality of frozen goat semen. *The Pharma Innovation Journal, 10*(7), 01-04.

Jenagrad, P.A., Kia, H.D., Moghaddam, G., & Ebrahimi, M. (2018). Evaluating caffeine antioxidant properties on Ghezel ram sperm quality after freeze-thawing. *Revue de Medecine Veterinaire, 169*(10-12), 233-40.

Lenzi, A., Gandini, L., Lombardo, F., Picardo, M., Maresca, V., Panfili, E.N.R.I.C.O., & Dondero, F. (2002). Polyunsaturated fatty acids of germ cell membranes, glutathione and blutathione-dependent enzyme-PHGPx: From basic to clinic. *Contraception, 65*(4), 301-304.

MacHugh, D.E., & Bradley, D.G. (2001). Livestock genetic origins: goats buck the trend. *Proceedings of the National Academy of Sciences, 98*(10), 5382-5384.

Shukla, M.K., & Misra, A.K. (2014). Caffeine as a semen additive to improve Murrah buffalo (Bubalus bubalis) semen cryopreservation. *Buffalo Bulletin, 33*(32), 6.

Singh, P., & Raina, V.S. (2000). Effect of caffeine, cAMP and cattle seminal plasma on freezability of buffalo bull semen. *Asian-Australasian Journal of Animal Sciences, 13*(7), 901-905.

Snedecor, G.W., & Cochran, W.G. (1994). *Statistical Methods*. 8th Edn., Iowa State University Press. Ames, Iowa, USA.

Spalekova, E., Makarevich, A.V., Kubovičová, E., Ostró, A., & Chrenek, P. (2014). Effect of caffeine on functions of cooling-stored ram sperm in vitro. *Acta Veterinaria Brno, 83*(1), 19-25.

Srivastava, S., & Kumar, S. (2014). Incorporation of ascorbic acid, caffeine and chloroquine diphosphate in dilutor improves structural and functional status of frozen semen. *Open Access Library Journal, 1*(1), 1-12.

Yashin, A., Yashin, Y., Wang, J.Y., & Nemzer, B. (2013). Antioxidant and antiradical activity of coffee. *Antioxidants, 2*(4), 230-245.