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Research Article

INCLUSION OF 1, 3, 7-TRIMETHYLXANTHINE IMPROVED SEMEN QUALITY PARAMETERS OF CATTLE BULL SEMEN

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ABSTRACT

Methylxanthines are phytochemicals that are obtained from plant secondary metabolism and are generated from the purine base xanthine. The most widely used methylxanthine is 1, 3, 7-trimethylxanthine (caffeine). The present study was undertaken to determine the effect of including 1, 3, 7-trimethylxanthine in a semen extender on sperm motility and morphological parameters of semen. Three adult cattle bulls were used to collect 15 semen ejaculates. Each ejaculate was divided into four groups. In group one (C), samples were diluted in Tris egg yolk glycerol extender, whereas the samples of other three groups (CF-1, CF-4 and CF-10) were diluted with Tris egg yolk glycerol extender containing addition of 1, 3 and 7-trimethylxanthine at dose rates of 1, 4 and 10 mM, respectively. Seminal parameters of the diluted semen samples were evaluated after being incubated at 37º C in a water bath for 15 and 30 minutes. Using a computer-assisted sperm analyzer (CASA), sperm motility, sperm kinetic parameters, and sperm abnormalities of semen from all groups were evaluated. The morphological characteristics of the spermatozoa in the supplemented and control groups were also assessed. Compared to the control and other supplemented groups, the results showed that spermatozoa motility, Kinematic parameters, viable sperms, and sperms with intact plasma membrane were significantly increased with the addition of 1, 3, 7-trimethylxanthine in a concentration of 4 mM. However, there was no statistically significant alteration in the sperms with bent tail, coiled tail, distal droplet, and distal midpiece reflex. Therefore, it can be concluded that impregnation of 1, 3, 7-trimethylxanthine (caffeine) in a semen extender at a concentration of 4 mM was able to enhance the quality of the semen.

Keywords: Methylxanthines, Semen, Semen extender, Motility, CASA

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The most common and widely used methods for breeding domestic animals are artificial insemination (AI) and semen cryopreservation (Jewgenow *et al.*, 2017) and AI using cryopreserved semen has played an instrumental role in the continuous progress of genetic improvement in livestock (Anzar, 2003). However, it has always been difficult to produce and supply frozen semen with a predicted fertility due to the low fertility of cryopreserved semen. In animal reproduction, semen extenders are preservation biological matrices that are used to help preserve life of sperm outside of the body for artificial insemination. To extend the life of sperm, these so-called extenders help protect and then nurture them and thereby increase the chances of a successful insemination. The total number of progressively motile spermatozoa in semen has a direct correlation with fertility, and sperm motility is one of the key factors that determines the quality of frozen thawed samples for AI (Mason, 2017). Changes in plasma membrane permeability in the tail region of spermatozoa and the formation of ice crystals in the mitochondria and axonemes following cryopreservation can both cause sperm motility to decrease (Prapaiwan *et al.*, 2016).

Methylxanthines are a special class of drugs derived from the purine base xanthine and have been shown to improve sperm motility and 1, 3, 7-trimethylxanthine (caffeine) is the most commonly used methylxanthine since it has greater lipophilic characteristics (Salihović *et al.*, 2014), which might contribute to its ability to easily diffuse through cell membranes and cross the blood-brain barrier (McCall *et al.*, 1982). Caffeine has been studied for its potential effects on sperm motility and viability. Some research has shown that caffeine may have a positive impact on sperm parameters and maintain their quality during the storage and transportation process in ram (Rateb *et al.*, 2020) and camel semen (El-Bahrawy, 2017). The addition of caffeine to the semen significantly increases the motility of sperm as a result of stimulation by cAMP and possibly decreasing apoptotic and dead/necrotic sperm. Sperm mitochondrial activity increases as a result of this rise in intracellular cAMP, and this is important since it provides energy for sperm movement (Li *et al.*, 2010). Hence, sperm motility is stimulated by cAMP via direct action on the axoneme of the tail or indirect action at the cell membrane as a secondary messenger (Garbers and Kopf, 1980). The effect of caffeine on sperm motility may depend on the species and may be dose- dependent (Špaleková *et al.*, 2014).

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During the process of cryopreservation, there is production of reactive oxygen species (ROS) and other intermediate free radicals. At physiological levels, ROS play a role in the regular functioning of sperm and serve as intracellular signalling molecules that are crucial for numerous physiological processes, including maturation, hyperactivation, capacitation, acrosome reaction (AR), and sperm-oocyte fusion (Aitken *et al.*, 1989). The presence of high ROS levels in seminal plasma lead to the lipid peroxidation of sperm plasma membranes, DNA damage of sperm and reduced antioxidant profile (Lone *et al.*, 2017), which in-turn result in death of many of the cells and altered characteristics of the remaining cells (Chatterjee and Gagnon, 2001). Lipid peroxidation levels correlate negatively with bull semen motility, plasma membrane integrity, and fertility (Kasimanickam *et al.*, 2007). It is necessary to maintain a balance between ROS production and antioxidant levels. Antioxidants may reduce cryodamage, decrease DNA fragmentation and early sperm maturation, shield sperm from ROS produced by abnormal sperm or leukocytes, and enhance sperm quality (Qamar *et al.*, 2022). According to studies, methylxanthines possess antioxidant properties. Caffeine effectively scavenges alkoxyl and hydroxyl radicals (León-Carmona and Galano, 2011) and this can support the antioxidant effect of caffeine in reducing lipid peroxidation thereby preventing cellular damage (Ofluoglu *et al.*, 2008). Thus, the study was planned to evaluate the effect of supplementation of 1, 3, 7-trimethylxanthine (caffeine) in semen extender on motility of sperm as well as morphological parameters of semen.

MATERIALS AND METHODS

Semen Collection and processing

For the study, 3-5-year-old, apparently healthy mature cow bulls weighing 450-550 kg and with a good libido were chosen. These animals were kept individually in semi-open sheds at the Animal Farm of the Department of Animal Genetics and Breeding, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The bulls were kept in a clean and sanitary environment with the same nutritional and managerial conditions with a balanced breeding bull diet and unlimited access to drinking water as per the Minimum Standard Protocol of the Government of India (2000). The bulls were worked out on a rotating bull exerciser before the semen collection. Semen was extracted twice a week in the morning using a sterile artificial vagina. The ejaculate was placed in a water bath at 37º C immediately after collection. A phase-contrast microscope with a warm stage (37º C) was used to subjectively evaluate sperm motility at 200x magnification. A total of 15 semen ejaculates with ≥70% sperm motility were used and each ejaculate was diluted at a concentration of 80 million sperm/mL using a TRIS egg yolk glycerol extender.

Extender preparation

TRIS-Egg yolk-Glycerol extender was prepared by adding 30.28 gm Tris, 16.75 gm citric acid, 12.00 gm D-Fructose in 1 litre of DDW. Egg yolk was added @ 20% and glycerol @ 7% to the volume of extender. The extender was supplemented with standard antibiotics (Penicillin @ 10 Lakh IU/litre and Streptomycin @ 1gm/litre). The diluted semen was divided into four aliquots, one aliquot (group C) was kept as control and the other three aliquots (groups CF-1, CF-4 and CF-10) were supplemented with 1, 4 and 10 mM 1, 3, 7-trimethylxanthine (caffeine), respectively in TEYG extender. The semen samples were kept in water bath at 37º C and samples were evaluated at 15 and 30 min of incubation for various kinematic and morphological parameters.

Evaluation of semen for kinetic parameters

Sperm kinematics, motility, and morphological anomalies were assessed using a computer-assisted sperm analyzer (CASA) system. Five optical fields were selected f or each sample on an eight-chamber Leja slide (depth 20 µm). Both fresh and frozen-thawed semen were analyzed using the CASA system (IVOS-II, Hamilton-Thorne Biosciences, Beverly, MA, USA) as described by Kumar *et al.* (2015). Before CASA analysis, the semen sample was diluted with pre-warmed Tris buffer to achieve a sperm concentration of approximately 40×10 sperm/mL. 2µL aliquot of the prepared semen was loaded onto a pre-warmed (38º C) eight-chamber Leja slide and analyzed for sperm kinetics and motility characteristics.

Total motility (TM, %), progressive motility (PM, %), average lateral head displacement (ALH, µm), average path distance (DAP), straight line distance (DSL), curvilinear distance (DCL), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), beat cross frequency (BCF, Hz), wobble (WOB), straightness (STR, %), and linearity (LIN, %) were all noted. Sperm abnormalities such distal droplet (%), bent tail (%), coiled tail (%), and distal midpiece reflex (%) were also observed. The CASA software settings were as follows: temperature = 38° C, frame rate = 60 Hz, frames acquired = 30, minimum contrast = 35, minimum cell size = 5 pixels, cell size = 9 pixels, cell intensity = 110 pixels, VAP cut-off = 37 µm/s, and STR cut-off = 50%.

Estimation of sperm livability and morphology

The viability and morphology of sperm was evaluated

using eosin-nigrosin staining technique as reported by Virmani *et al.* (2020). The eosin-nigrosin stain was heated to 37º C for 30 minutes using a hot air oven. Following a thorough mixing of semen sample and the stain, a drop was smeared on a heated, grease-free glass slide and the smear was left to air dry at room temperature. A 1000X magnification microscope was used to view the slides. Live spermatozoa remained unstained while dead spermatozoa took pink red stain against blue-black background, partially stained sperms counted as dead. Approximately 200 sperm randomly from different fields were counted and percent live-dead and sperm morphology was calculated.

Estimation of acrosome integrity

Giemsa stain was used to determine the percent intact acrosome as per the method reportedby Singh *et al.* (2020). A thin smear of extended semen was prepared, dried and fixed in neutral formalin saline solution for 15 min. The smear was thoroughly rinsed with running tap water, and then stained for 40 minutes in a working Giemsa stain solution. A phase contrast microscope (1000X) was used to assess the degree of acrosome reaction. About 200 sperm were counted from different fields to calculate the percentage of sperm with intact acrosomes.

Assessment of plasma membrane integrity

Evaluation of the functional integrity of sperm membrane was done by Hypo-osmotic swelling test (HOST) as per the method described by Kumar *et al.* (2015). HOST is better indicator of fertilizing capacity of sperm than super vital staining. Assay was performed by mixing 100 µL of semen sample with 1 mL hypo-osmotic solution (0.735 g sodium citrate $2H_2O$ and 1.351 g fructose in 100 mL distilled water). Sperm tail bending/coiling was assessed after 60 min incubation at 37º C by placing a 15 µL aliquot of the well-mixed sample incubated at 37º C on a warm slide examined under light microscopy (400X magnification). At least 200 spermatozoa were observed per slide. Sperm with coiled tail after incubation was considered having intact plasma membrane.

Statistical Analysis

The data were analyzed using the SPSS (Version 23) software package. The sperm quality parameters were compared in control and supplemented groups using one-way analysis of variance. Mean differences were examined employing Duncan's multiple range tests. Variation was deemed significant when $P \leq 0.05$. The values for all parameters were given as mean ±standard error.

RESULTS AND DISCUSSION

Significant variables in sperm fertilization activities include motility, vitality rates, and sperm count, all of which are crucial in influencing the rate of fertilization (Homonnai *et al.*, 1976). Numerous studies have been conducted to improve the ability and capability of sperms and qualitative and quantitative improvement of laboratory fertilization. During the present study, effect of caffeine supplementation was evaluated for sperm attributes in cattle bull semen stored at 37º C. Numerous studies have been conducted to improve the quality and quantity of laboratory fertilization as well as the ability and aptitude of sperm. The values recorded for motile sperm as well as progressive motile sperms were significantly ($p<0.05$) higher in CF-4 group as compared to other groups evaluated during the experiment (Table 1). The higher values seen in CF-4 could be attributed to the fact that caffeine increases cAMP levels inside of cells via regulating phosphodiesterase, an enzyme that analyzes cAMP (Anahita *et al.*, 2005). All supplemented groups showed non-significant differences in sperm abnormalities parameters such as bent, coiled, and distal droplet and distal midpiece reflex after 15 and 30 minutes of incubation, when compared to the control group.

Sperm actual kinematic parameters *viz*., ALH, DAP, DCL, DSL, VAP, VCL, VSL and BCF were measured using CASA. ALH showed non-significant variation during the first reading i.e., at 15 min interval. However there was significant increase in the value at 30 min incubation in CF-1 and CF-4 groups. But at higher concentration in CF-10 group, the values decreased than control samples. DAP, DCL and DCL showed non-significant increase in groups supplemented with caffeine at lower doses. VAP values were significantly higher in CF-4 group at 15 min of incubation and in CF-1 and CF-4 groups at 30 min of incubation. Similarly VCL and VSL showed significant increase in CF-1 and CF-4 groups as compared to control and CF-10 groups. BCF values varied non-significantly among the control and supplemented groups. Mitochondria play a crucial role in providing energy to sperm and axoneme serves as the structural basis of the sperm tail responsible for propulsion, is vital for sperm motility. Any damage to these organelles could hinder the sperm's ability to move efficiently (Ricci *et al.*, 2017). The decline in the velocity related parameters of sperms after incubation with higher dose of caffeine in CF-10 might be due to damage of mitochondrial apparatus and axoneme of sperm. The results of the present study corroborated with the findings of Špaleková *et al.* (2011 and 2014). They studied the effect of supplementing different concentrations (1-4 mM) of caffeine in ram semen *in vitro* and observed significant increase in sperm motility and progressive movement that maintained in a

Table 1. Effect of supplementation of caffeine on sperm motility and sperm abnormalities after 15 and 30 min of incubation at 37° C

Parameter	Incubation Time (min)	C	CF-1	CF-4	CF-10	p value
Motile sperm (%)	15	47.58±4.53	59.91±3.34	60.62±4.57	52.07±5.07	0.120
	30	51.09^{a}±3.39	54.39^{a}±3.42	66.23^{b}±4.63	52.74^{a}±4.85	0.050
Progressive motile sperm (%)	15	34.17±3.62	40.31±4.42	43.64±5.37	33.51±3.79	0.295
	30	35.15^{a}±2.78	35.15^{a}±4.42	46.83^{b}±4.78	28.24^{a}±3.24	0.050
Sperm Abnormalities						
Bent tail (%)	15	0.29±0.09	0.31±0.16	0.35±0.21	0.39±0.17	0.968
	30	0.27±0.16	0.25±0.11	0.16±0.05	0.74±0.41	0.275
Coiled tail (%)	15	0.01±0.01	0.07±0.07	0.04±0.02	0.01±0.01	0.673
	30	0.01±0.01	0.00±0.00	0.00±0.00	0.05±0.04	0.112
Distal droplet (%)	15	0.07±0.04	0.00±0.00	0.19±0.19	0.05±0.04	0.575
	30	0.04±0.03	0.02±0.01	0.03±0.03	0.05±0.04	0.849
Distal midpiece reflex (%)	15	6.44±0.89	3.63±0.49	4.43±0.52	4.82±0.57	0.023
	30	4.03±0.71	4.53±0.63	6.04±0.65	5.89±0.90	0.147

Mean values (Mean ± SE) with different superscript in a row differ significantly ($p<0.05$)
CF-1: Caffeine 1mM; CF-4: Caffeine 4mM and CF-10: Caffeine 10 mM

Table 2. Effect of supplementation of Caffeine on actual kinematic parameters of spermatozoa after 15 and 30 min of incubation at 37° C

Parameter	Incubation Time (min)	C	CF-1	CF-4	CF-10	p value
ALH (µm)	15	9.39±0.67	8.27±0.82	8.58±0.77	7.75±0.47	0.413
	30	5.70^{ab}±0.25	6.78^{b}±0.70	6.53^{b}±0.59	4.66^{a}±0.28	0.016
DAP (µm)	15	25.98±1.26	22.42±1.60	26.25±1.94	23.40±1.58	0.258
	30	22.25±1.35	24.06±2.52	24.29±1.51	18.71±1.26	0.097
DCL (µm)	15	50.24±3.49	42.26±3.87	47.65±4.09	45.58±3.58	0.497
	30	37.64±1.98	42.73±5.01	41.83±3.07	31.01±2.31	0.061
DSL (µm)	15	20.31±0.85	18.12±1.15	21.73±1.69	18.21±1.24	0.139
	30	19.13±1.33	20.30±2.06	20.81±1.44	16.27±1.13	0.166
VAP (µm/s)	15	105.19^{b}±6.26	92.57^{ab}±8.55	105.20^{b}±8.87	77.57^{a}±4.42	0.027
	30	75.06^{ab}±2.63	86.83^{b}±9.43	81.37^{b}±6.12	59.43^{a}±3.29	0.013
VCL (µm/s)	15	200.81±14.54	175.96±18.29	192.31±17.97	148.61±10.01	0.100
	30	125.78^{ab}±4.45	153.29^{b}±18.36	142.93^{b}±13.69	98.71^{a}±6.1o	0.013
VSL (µm/s)	15	82.51b±4.33	74.47^{ab}±6.03	87.12^{b}±7.32	60.57^{a}±3.17	0.006
	30	64.93^{ab}±2.75	73.66^{b}±7.67	69.08^{b}±5.08	51.44^{a}±2.82	0.018
BCF (Hz)	15	23.06±0.62	23.31±0.53	24.58±0.54	22.84±0.52	0.130
	30	27.29±0.64	26.66±0.38	25.72±0.56	27.09±0.33	0.128

Mean values (Mean ± SE) with different superscript in a row differ significantly ($p<0.05$)
CF-1: Caffeine 1mM; CF-4: Caffeine 4mM and CF-10: Caffeine 10 mM

dose dependent manner during cooling storage for up to 72 h. In another study, Lecewicz *et al.* (2019) supplemented canine semen with different concentrations of caffeine and revealed that stimulatory and significant effect of caffeine at 10 mM concentration on total and progressive motility. Srivastava and Kumar (2014) while investigating on semen reported that caffeine (0.54 mM) significantly improved the semen motility parameters, especially the progressive motility. It has been demonstrated in various mammalian species that calcium is crucial for sperm capacitation (Yanagimachi, 1994) and hyperactivated flagella have been reported to be associated with an increase in intracellular Ca^{2+} concentration (Ho and Suarez, 2001).

Table 3. Effect of supplementation of Caffeine on relative kinematic parameters of spermatozoa after 15 and 30 min of incubation at 37º C

Parameter	Incubation Time (min)	C	CF-1	CF-4	CF-10	p value
WOB (%)	15	56.25±1.97	58.51±3.41	60.34±2.8	57.00±1.83	0.690
	30	62.24±1.14	61.05±1.43	63.33±2.90	66.63±1.88	0.223
LIN (%)	15	46.14±2.59	50.10±4.22	52.18±3.63	47.04±2.39	0.547
	30	54.79±1.70	53.47±1.88	56.26±3.54	60.12±2.46	0.276
STR (%)	15	80.20±1.71	83.40±1.95	83.98±2.00	80.05±1.63	0.289
	30	85.55±1.56	85.47±1.07	86.27±1.37	87.46±1.48	0.724

Mean values (Mean ± SE) with different superscript in a row differ significantly ($p<0.05$)
CF-1: Caffeine 1mM; CF-4: Caffeine 4mM and CF-10: Caffeine 10 mM

Table 4. Effect of supplementation of Caffeine on morphological parameters of spermatozoa after 15 and 30 min of incubation at 37º C

Parameter	C	CF-1	CF-4	CF-10	p value
Acrosome integrity (%)	79.67^{a}±1.28	81.13^{ab}±1.26	84.67^{b}±1.54	81.07^{ab}±1.39	0.076
Viability (%)	62.00^{a}±0.56	62.47^{a}±0.40	65.27^{b}±0.84	63.07^{a}±0.65	0.003
Plasma membrane integrity	61.13^{a}±0.42	62.80^{ab}±0.45	64.53^{b}±1.15	61.13^{a}±0.45	0.002

Mean values (Mean ± SE) with different superscript in a row differ significantly ($p<0.05$)
CF-1: Caffeine 1mM; CF-4: Caffeine 4mM and CF-10: Caffeine 10 mM

Further, the protection incurred by caffeine at different levels was evaluated on plasma membrane through assessment of sperm with normal plasma membrane fluidity, acrosomal integrity and HOST response which exhibited significantly ($p<0.05$) higher values in the CF-4 group (Table 3). Spermatozoa with intact acrosome increased significantly in all the supplemented groups as compared to control group, however the rise was maximum in CF-4 group. Livability and plasma membrane integrity was observed to be increased significantly in samples of CF-4 group in comparison to all other groups. Several studies have also reported that adding caffeine to the extender significantly ($p<0.05$) increase acrosome integrity and/or HOS reactivity in semen of buffalo bull (Singh and Raina, 2000; Shukla *et al.*, 2014), HF bull (Srivastava and Kumar, 2014), and ram (Jenagrad *et al.*, 2018; Rateb *et al.*, 2020). Additionally, studies conducted on cattle bull and buck semen by Pereira *et al.* (2000), ram semen by Abd El-Hamid (2019), and camel semen by El-Bahrawy (2017) all demonstrated the beneficial effects of caffeine on the percentage of acrosome integrity.

A significant amount of the sperm must have intact membrane for fertilization to be successful (Špaleková *et al.*, 2014). The "acrosome reaction" occurs when spermatozoa release their acrosomal contents to penetrate the extracellular matrix of the egg and get to the plasma membrane of the oocyte during fertilization. demonstrated that coffee has a favourable impact on the percentage of acrosome integrity. Therefore, increasing the number of sperm with intact acrosomes at the site of fertilization helps to increase the rate of conception (Gadella and Evans, 2011). According to studies, caffeine increases acrosome reaction and/or capacitation of spermatozoa. When added to the fertilization medium, caffeine instantaneously hyperactivates sperm by raising intracellular calcium levels (Colás *et al.*, 2010). Additionally, studies in pigs (Nagai *et al.*, 1993) and mice (Fraser, 1979) have shown that caffeine accelerates sperm penetration *in vitro*.

CONCLUSION

From the study, it can be concluded that enrichment of cattle semen with lower concentration of 1, 3, 7-trimethylxanthine (Caffeine) enhances its sperm motility, live sperm percentage, acrosome integrity and when compared to control. But caffeine at 4 mM concentration showed significant improvement in the semen quality as compared to control and other supplemented groups. Thus, the optimum dose of 1, 3, 7-trimethylxanthine (caffeine) is 4 mM for further use in semen extender, however additional research on *in vitro* and *in vivo* validation of treated semen is required in order to evaluate the fertilizing ability of semen.

REFERENCES

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. *Reprod. Domest. Anim.* **54(2)**: 225-233.

Aitken, R.J., Clarkson, J. and Fishel, S. (1989). Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol. Reprod.* **41**: 183–197.

Anahita, V., Asilian, A., Khalesi, F., Khodami, L. and Shahtalebi, M.A. (2005). Caffeine effect in treatment of pezoriazisvolgaris: randomal clinical experimental work. *Seasonal J. Skin Dis.* **181**: 465-462.

Anzar, M., Farooq, U., Mirza, M.A., Shahab, M. and Ahmad, N. (2003). Factors affecting the efficiency of artificial insemination in cattle and buffalo in Punjab, Pakistan. *Pak. Vet. J.* **23(3)**: 106-113.

Chatterjee, S. and Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol. Reprod. Dev.* **59(4)**: 451-458.

Colás, C., Cebrián-Pérez, J.A. and Muiño-Blanco, T. (2010). Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. *Int. J. Androl.* **33(1)**: 187-197.

El-Bahrawy, K., Rateb, S., Khalifa, M., Monaco, D. and Lacalandra, G. (2017). Physical and kinematic properties of cryopreserved camel sperm after elimination of semen viscosity by different techniques. *Anim. Reprod. Sci.* **187**: 100-108.

Fraser, L.R. (1979). Accelerated mouse sperm penetration *in vitro* in presence of caffeine. *J. Reprod. Fertil.* **57**: 337-384.

Gadella, B.M. and Evans, J.P. (2011). Membrane fusions during mammalian fertilization. *Adv. Exp. Med. Biol.* **1**: 65-80.

Garbers, D. and Kopf, G. (1980). The regulation of spermatozoa by calcium and cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* pp. 251-306.

Ho, H.C. and Suarez, S.S. (2001). Hyperactivation of mammalian spermatozoa: function and regulation. *Reprod.* **122(4)**: 519-526.

Homonnai, Z.T., Paz, G., Sofer, A., Kraicer, P.F. and Harell, A. (1976). Effect of caffeine on the motility, viability, oxygen consumption and glycolytic rate of ejaculated human normokinetic and hypokinetic spermatozoa. *Int. J. Fertil.* **21(3)**: 162-170.

Jewgenow, K., Braun, B.C., Dehnhard, M., Zahmel, J. and Goeritz, F. (2017). Research on reproduction is essential for captive breeding of endangered carnivore species. *Reprod. Domest. Anim.* **52**: 18-23.

Kasimanickam, R., Kasimanickam, V., Thatcher, C.D., Nebel, R.L. and Cassell, B.G. (2007). Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters and competitive index in dairy bulls. *Theriogenol.* **67(5)**: 1004-1012.

Kumar, P., Kumar, D., Sikka, P. and Singh, P. (2015). Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. *Anim. Reprod. Sci.* **152**: 26-31.

Lecewicz, M., Strzeżek, R., Majewska, A.M., Purpurowicz, P.S. and Kordan, W. (2019). The effect of different concentrations of caffeine, pentoxifylline and 2'-deoxyadenosine on the biological properties of frozen-thawed canine semen. *Ann. Anim. Sci.* **19(3)**: 733-746.

León-Carmona, J.R. and Galano, A. (2011). Is caffeine a good scavenger of oxygenated free radicals? *J. Phys. Chem. B.* **115(15)**: 4538-46.

Li, P., Li, Z.H., Dzyuba, B., Hulak, M., Rodina, M. and Linhart, O. (2010). Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio* L.) sperm caused by cryopreservation techniques. *Biol. Reprod.* **83(5)**: 852-858.

Lone, S.A., Shah, N., Yadav, H.P., Wagay, M.A., Singh, A. and Sinha, R. (2017). Sperm DNA damage causes, assessment and relationship with fertility: A review. *Theriogenol. Insight.* **7(1)**: 13-20.

Mason, S.J. (2017). A retrospective clinical study of endoscopic-assisted transcervical insemination in the bitch with frozen–thawed dog semen. *Reprod. Domest. Anim.* **52**: 275-280.

McCall, A.L., Millington, W.R. and Wurtman, R.J. (1982). Blood-brain barrier transport of caffeine: Dose-related restriction of adenine transport. *Life Sci.* **31**: 2709-2715.

Nagai, T., Miura, K., Kikuchi, K. and Okamura, N. (1993). Effects of caffeine on *in vitro* fertilization of pig follicular oocytes. *J. Reprod. Dev.* **39**: 347-352.

Ofluoglu, E., Pasaoglu, H. and Pasaoglu, A. (2009). The effects of caffeine on L-arginine metabolism in the brain of rats. *Neurochemical Res.* **34**: 395-399.

Pereira, R.J.T.A., Tuli, R.K., Wallenhorst, S. and Holtz, W. (2000). The effect of heparin, caffeine and calcium ionophore A 23187 on *in vitro* induction of the acrosome reaction in frozen-thawed bovine and caprine spermatozoa. *Theriogenol.* **54(2)**: 185-192.

Prapaiwan, N., Tharasanit, T., Punjachaipornpol, S., Yamtang, D., Roongsitthichai, A., Moonarmart, W. and Manee-In, S. (2016). Low-density lipoprotein improves motility and plasma membrane integrity of cryopreserved canine epididymal spermatozoa. Asian-Australas. *J. Anim. Sci.* **29(5)**: 646.

Qamar, A.Y., Naveed, M.I., Raza, S., Fang, X., Roy, P.K., Bang, S., Tanga, BM, Saadeldin, I.M., Lee, S. and Cho, J. (2023). Role of antioxidants in fertility preservation of sperm- A narrative review. *Anim. Biosci.* **36(3)**: 385-403.

Rateb, S.A., Khalifa, M.A., Abd El-Hamid, I.S. and Shedeed, H.A. (2020). Enhancing liquid-chilled storage and cryopreservation capacities of ram spermatozoa by supplementing the diluent with different additives. *Asian-Australas. J. Anim. Sci.* **33(7)**: 1068.

Ricci, E., Viganò, P., Cipriani, S., Somigliana, E., Chiaffarino, F., Bulfoni, A., Parazzini, F. (2017). Coffee and caffeine intake and male infertility: A systematic review. *Nutr. J.* **16(1)**: 37.

Salihović, M., Huseinović, S., Špirtović-Halilović, S., Osmanović, A., Dedić, A., Ašimović, Z. and Završnik, D. (2014). DFT study and biological activity of some methylxanthines. *Bull. Chem. Technol. Bosnia Herzeg*. **42**: 31-36.

Shukla, K.K., Chambial, S., Dwivedi, S., Misra, S. and Sharma, P. (2014). Recent scenario of obesity and male fertility. *Androl.* **2(6)**: 809-818.

Singh, G.V., Kumar, A., Sachan, V., Agrawal, J.K. and Saxena, A. (2020). Effect of the addition of insulin like growth factor-I (IGF-I) on cryopreservation of Hariana bull semen. *Iran. J. Appl. Anim. Sci.* **10(4)**: 611-614.

Singh, P. and Raina, V.S. (2000). Effect of caffeine, cAMP and cattle seminal plasma on freezability of buffalo bull semen. *Asian-Australas. J. Anim. Sci.* **13(7)**: 901-905.

Špaleková, E., Makarevich, A. and Pivko, J. (2011). Effect of caffeine on parameters of ram sperm motility. *Slovak. J. Anim. Sci.* **44(2)**: 78-83.

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

Srivastava, S. and Kumar, S. (2014). Incorporation of ascorbic acid, caffeine and chloroquine diphosphate in dilutor improves structural and functional status of frozen semen. *O.A. Lib. J.* **1(1)**: 1-12.

Virmani, M., Malik, R.K., Singh, M., Patil, C.S., Singh, P. and Vijayalakshmy, K. (2020). Enrichment of semen extender with Syzygiumaromaticum extract: its impact on bull sperm preservation as an alternative to antibiotics. *Ruminant Sci.* **9(1)**: 63-68.

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. *Zygote.* **2(4)**: 371-372.