Evaluating caffeine antioxidant properties on Ghezel ram sperm quality after freeze-thawing

P. ALIPOUR JENAGRAD, H. DAGHIGH KIA*, G. MOGHADDAM, M. EBRAHIMI

Introduction

Semen cryopreservation is a unique technique for preserving genetic diversity, especially endangered species [10]. Though, oxidative stress and cold shock along with increasing cAMP permeability cause an impairment in post-thaw spermatozoa motility and lifespan [49, 10]. High concentration of polyunsaturated fatty acids in ram sperm membranes and limited enzymatic antioxidant defense system [such as: glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase] make them more susceptible to oxidative stress damages induced by reactive oxygen species (ROS) especially during freeze-thawing [8]. Accordingly, a pile of researches focused on counteracting the reeve effects of ROS on sperm fertility using different types of antioxidants [5, 6].

Caffeine (1,3,7-tri-methylxanthine) is in a methylxanthine group with antioxidant properties [29, 53, 37]. Supplementing methylxanthines into the sperm suspension improved sperm motility and velocity movements in fresh or freeze-thawed spermatozoa [35, 24, 46]. Moreover, methylxanthines decreased acrosomal loss during the freeze-thawing process and increased sperm mitochondrial activity which plays a key role in providing energy for sperm motility [29]. Methylxanthines can also enhance sperm fertility by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase enzyme and then cause depositing sperm intracellular cAMP which plays an important role in sperm physiology and motility [29, 12]. Methylxanthines have also powerful scavenging capacity which protect sperm from membrane damages and DNA fragmentation mediated by ROS during freeze-thawing [18]. In a study, caffeine improved bovine sperms’ respiration and their motility [22]. Moreover, in vivo studies showed increasing effect of caffeine produces on serum testosterone levels along with higher sperm concentration, preservability, and post-thaw motility

SUMMARY

To reduce oxidative damages during freeze-thawing on sperm fertility, this study was designed to investigate the antioxidant effect of caffeine in a soybean lecithin extender on the performance of freeze-thawed sperm of Ghezel rams. Accordingly, semen of 5 Ghezel rams was collected twice a week and Tris based extender (1.5% lecithin and 7% glycerol) was used to dilute samples accompanied by adding 0, 0.5, 1, 2, and 4 mM/L caffeine levels. Semen samples were then cooled, frozen, and kept in a liquid nitrogen. After thawing the samples at 37°C, semen performance was evaluated including: sperm motility, plasma membrane integrity, morphology, viability, membrane lipid peroxidation, total antioxidant capacity, and the activity of glutathione peroxidase and superoxide dismutase. Finally, data were analyzed using GLM procedure of SAS software. Results indicated an increase in sperm velocity parameters by adding 2 mM/L caffeine compared with the other groups (P<0.05). Also, a decrease in abnormal sperm percentage was observed by adding 2 mM/L caffeine (P<0.05). Moreover, adding 1 mM/L caffeine improved sperm viability rate, glutathione peroxidase activity, and total antioxidant capacity compared with other groups (P<0.05). The overall results of this study showed that adding 1 and 2 mM/L caffeine in Tris-lecithin based extender can improve the quality characteristics of freeze-thawed semen.

Keywords: Caffeine, Freeze-thawing process, Ghezel ram, Sperm quality

RéSUMÉ

Évaluation des propriétés antioxydantes de la caféine sur la qualité du sperme de bélier Ghezel après congélation-décongélation

Cette étude a été conçue pour étudier l'effet antioxydant de la caféine dans un extenseur de lécithine de soja pendant la congélation-décongélation des spermatozoïdes des béliers Ghezel. Le sperme de 5 béliers Ghezel a été recueilli deux fois par semaine et dilué dans un extenseur à base de Tris (1,5 % de lécithine et 7 % de glycérol) contenant 0, 0,5, 1, 2 et 4 mM/L de caféine. Les échantillons de sperme ont ensuite été refroidis, congelés, conservés dans de l’azoté liquide. La motilité des spermatozoïdes, l’intégrité de la membrane plasmatique, la morphologie, la viabilité, la peroxydation des lipides membranaires, la capacité antioxydante totale et l’activité du glutathione peroxydase et du superoxyde dismutase ont été évaluées après décongélation des échantillons à 37°C. Enfin, les données ont été analysées à l’aide de la procédure GLM du logiciel SAS. Les résultats ont indiqué une augmentation des paramètres de la vitesse des spermatozoïdes en ajoutant 2 mM/L de caféine par rapport aux autres groupes (P<0,05). Une diminution du pourcentage de spermatozoïdes anormaux a été observée en ajoutant 2 mM/L de caféine (P<0,05). Le taux de viabilité des spermatozoïdes, l’activité glutathion peroxydase et la capacité antioxydante totale ont été améliorés par rapport aux autres groupes, en ajoutant 1 mM/L caféine (P<0,05). Les résultats globaux de cette étude ont montré que l’ajout de 1 et 2 mM/L de caféine dans un extenseur à base de tris-lecithine peut améliorer la qualité et les caractéristiques du sperme congélée-décongelé.

Mots-clés : Caféine, congélation, bélier, sperme, Ghezel
Sperm abnormalities were evaluated by Hancock test after.

Straw), and were sealed with haematocrit putty. In the IMV, L'Aigle, France, (4 × 10^6/mL) were then loaded into 0.25 mL French straws containing different concentration of caffeine (0, 0.5, 1, 2, and 4 mM/L). Afterwards, diluted treatment semen samples were gradually cooled at 4°C for 2 h. semen samples were then loaded into 0.25 mL French straws (IMV, L'Aigle, France), (4 × 10^6/mL, spermatozoa per straw), and were sealed with haematocrit putty. In the next step, the sealed straws were horizontally frozen by liquid nitrogen vapors (6 cm above liquid nitrogen for 10 min) and then immersed into liquid nitrogen (-196°C) for storage [7]. For post thaw quality evaluations, the frozen straws were thawed individually in a water bath at 37°C for 30 s.

EVALUATION SPERM PARAMETERS AFTER THAWING SPERM MOTILITY AND VELOCITY PARAMETERS BY CASA

Immediately after thawing, a computer-assisted sperm motility analysis (CASA, Animal Version 12.3 CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to record ram sperm motility and velocity characteristics using a phase-contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA, USA) included: progressive motility (PM, %), total motility (TM, %), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), and linearity (LIN, %). For this reason, freeze-thawed semen samples (10 μL) were placed on a prewarmed slide and covered with a cover slip. Then, samples were examined at magnification ×10 (negative phase-contrast) under the microscope with a warmed stage (37°C). For each evaluation, at least five microscopic fields or a total of 200 spermatozoa were analyzed.

SPERM VIABILITY

Semen sperm viability was examined using eosin-nigrosin staining method. Eosin-nigrosin staining liquid composed of 1.67 g eosin-Y, 10 g nigrosin, and 2.9 g sodium citrate which dissolved in 100 mL distilled water. For this reason, a small droplet of semen suspension (10 μL) and a larger droplet of stain (20 μL) were mixed and spread on a clean prewarmed (37°C) glass slide. After drying the samples, viability was determined by counting 200 spermatozoa at the magnification of 400× under phase-contrast microscope (CKX41; Olympus, Tokyo, Japan). Sperms which displayed partial or complete purple staining were considered non-viable, while completely clear sperms considered viable [43, 36] (Figure 1).

Figure 1: Sperm abnormalities were evaluated by Hancock test
SPERM PLASMA MEMBRANE INTEGRITY

Sperm membrane functional integrity was evaluated after freeze-thawing by hypo-osmotic swelling (HOS) test which was based on curled and swollen tails (Figure 2). For this reason, 10 μL of centrifuged semen sample (1200×g for 10 min at 20°C) was added to 200 μL of hypo-osmotic solution (100 mOsm / kg, 9 g/L fructose, 4.9 g/L sodium citrate, and 100 mL distilled water) and incubated for 60 min at 37°C. Afterward, 10 μL of this mixture was placed on a warm glass slide and covered with a coverslip, then 200 spermatozoa were evaluated under microscope 400×magnification to determine sperms with coiled and swollen tails [43, 17].

SPERM ABNORMALITIES

Hancock solution was applied to assess sperm abnormalities of semen samples (Schafer and Holzmann, 2000), (Figure 3). Hancock solution mixture was prepared by adding 150 mL of sodium saline solution (9.01 g NaCl in 500 mL of distilled water), 62.5 mL formalin (37% formaldehyde), 500 mL of double-distilled water and 150 mL of buffer solution. Buffer solution (280 mL) was made by blending 200 mL of solution A (21.7 g Na2HPO4 × H2O in 500 mL of distilled water) and 80 mL of solution B (22.254 g KH2PO4 in 500 mL of distilled water). Then, 150 μL of Hancock solution and 15 μL of each thawed semen sample were mixed and 10 μL of this mixture were placed on a glass slide and evaluated for sperm abnormalities under microscope at 400× magnification.

BIOCHEMICAL ASSAY MALONDIALDEHYDE (MDA) CONCENTRATIONS

The concentration of MDA (as a marker of lipid peroxidation) in semen samples were evaluated using thiobarbituric acid reaction [18]. First, 1mL of diluted semen sample (250×10⁶ spermatozoa/mL) was added to 1 mL of cold 20% (wt/vol) trichloroacetic acid. Second, 1 mL of butylated hydroxytoluene (2% BHT solved in ethanol) and 1 mL of EDTA (1 mM concentration) were added to the previous mixture to inhibit lipid oxidation. Then, mixture was centrifuged (1200 ×g for 15 min) and 1 mL of supernatant incubated with 1 mL of 0.67% (wt/vol) thiobarbituric acid in a boiled water bath (100°C for 10 min). Soon after cooling, absorbance of samples was recorded using a spectrophotometer (T80 UV/VIS PG Instrument Ltd, UK) based on 532 nm and results represented as nM/mL.

SEMINAL PLASMA GPX ACTIVITY

Seminal plasma GPx activity was evaluated using a commercial kit (Randox, Crumlin, UK) using the manufacture’s manual. Then, the absorbance of samples was recorded at 340 nm using spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) and GPx activity was reported as mU/mL in seminal plasma samples [41].

SEMINAL PLASMA SOD ACTIVITY

Seminal plasma SOD activity was determined using a commercial kit (Randox, Crumlin, UK) using the manufacturer’s manual. Then, SOD activity was recorded by a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) with the absorbance of 505 nm and reported as U/mL in seminal plasma samples [41].

TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity was measured using a commercial kit (Randox, Crumlin, UK) using a
spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) with the absorbance of 600 nm and reported as mM/L in seminal plasma samples [41].

STATISTICAL ANALYSIS

The present experiment was designed based on a completely randomized design with 5 treatments (caffeine levels) and 5 replicates. Before analysis, all data were assessed for normality using Proc Univariate and arc-sin transformation used for data with the lack of normal distribution. Then, data were analyzed by GLM procedure of SAS (Ver. 9.4, 2015). Tukey-Kramer procedure was used for multiple comparison of means with the confidence level of 0.05. The statistical model are presented below:
other hand, no difference (P>0.05) was observed between 4 mM/L caffeine treatment and the control group and even numerical increase in total spermatozoa abnormality was observed in this treatment group.

The percentage of spermatozoa viability was also significantly increased by all caffeine treatments (P<0.01) and the highest amount of spermatozoa viability was observed in 1 mM/L and 4 mM/L caffeine treatments (Table 2).

Based on the results, both GPx and total antioxidant capacity of semen were affected by adding different concentrations of caffeine in a Tris based extender (P<0.01) and the highest amount was observed in 1 mM/L caffeine treatment (Table 2).

Present results showed no effect of caffeine treatments on membrane integrity, MDA, and SOD (P>0.05), (Table 2).

Discussion

Semen cryopreservation is a well established technique for preserving spermatozoa, though freeze-thawing process reduces sperm motility and viability, damages mitochondria, plasma and acrosome membranes, while increases DNA fragmentation and oxygen free radicals [48, 37]. Also, high content of polyunsaturated fatty acids in spermatozoa makes it vulnerable to oxidative stress and lipid peroxidation during cryopreservation and increases malondialdehyde production, which considered as an oxidative stress marker [24, 50, 8]. Meanwhile, it was indicated that cold shock causes structural damage in plasmalemma which in turn causes leakage of cAMP into the extracellular medium [49]. Accordingly, variety of substances included methylxanthines added to sperm suspensions to improve fresh and cryopreserved sperm motility characteristics [21, 23]. In the present study, caffeine as a methylxanthine derivative was supplemented to soybean lecithin-based semen extender in order to improve freeze-thawed ram spermatozoa quality characteristics.

It was indicated that motility parameters are important for freeze-thawed ram spermatozoa function and fertility [27]. Based on the present results, adding 2 mM/L (0.5 mM/L for BCF) caffeine in a Tris-based extender before cryopreservation had the best improving effect on velocity parameters (VAP, VSL, VCL, BCF and LIN) of spermatozoa, while no effect of caffeine treatments was observed on TM, PM, and STR. Another study indicated higher amount of VAP, VSL, VCL, and ALH (after four hours of incubation), while lower or no effect of caffeine on TM and PM of frozen/thawed bovine spermatozoa [11]. Higher TM and PM until 72 h of cooling-storage at 4-5°C by adding caffeine in a Triladyl extender was reported in ram spermatozoa [45]. Also, it was indicated that adding caffeine in diluent after freeze thawing of Buffalo semen improved post-thawed motility more than using caffeine in diluent before freezing [20, 45]. Furthermore, adding caffeine to diluent increased sperm motility of buffalo bull semen [45]. It was indicated that adding caffeine before cryopreservation or immediately after thawing causes an increase in semen post-thaw motility [11]. It was proved that caffeine induces spermatozoa hyper activation by increasing in calcium content of spermatozoa rather than increasing in intracellular cAMP level [13]. Moreover, other studies indicated the role of methylxanthines on inhibiting cAMP phosphodiesterase, which causes an increase in cellular content of cAMP [23]. As a second messenger, cAMP plays a crucial role in signal transduction [32] which improves sperm motility and metabolism (simulates glycolysis), [40, 43], the passage of calcium ions from the plasma membrane [39] along with sperm acrosomal reaction and capacitation [21].

Since healthy and intact plasma membrane are important for sperm viability and fertilization, Hypo-osmotic swelling test (HOST) was used for evaluating membrane integrity of ram spermatozoa [30, 3]. Though results of the present study demonstrated no significant effect of adding different concentrations of caffeine on plasma membrane integrity. The present results was in agreement with the results of Spalekova et al. [46], while in the opposite with the results of Srivastava and Kumar [47]. Also, a previous study reported no effect of adding caffeine in diluents on acrosomal damages of spermatozoa during freezing process [45].

Results of the present study showed a decreasing effect of caffeine (especially in 2 mM/L caffeine treatment) on spermatozoa total abnormality. In a previous study, caffeine reduced buffalo bull sperm abnormality before or immediately after freezing [45]. Also, adding green tea (Camellia sinensis) extract in a soybean lecithin extender did not have any effect on spermatozoa abnormality percentage of post-thawing ram semen [33].

It was indicated that preserving sperm fertility depends on its viability [15]. Results of the present study indicated positive effects of caffeine on spermatozoa viability, especially in 1 mM/L and 4 mM/L caffeine treatments. Resemble to the present results, a previous study indicated the positive effect of caffeine on maintaining viability, while reducing occurrences of apoptotic spermatozoa in cooled-stored ram semen [46]. Also, it was indicated that supplementing sperm storage medium with caffeine improved rat sperm viability [15].

Sperm membrane lipid peroxidation considered as an affective indicator of sperm preservance [8]. Although ROS production has some improving effects on fertilization, over production of ROS can usually impair permeability and fluidity of the plasma membrane and...
reduces sperm viability [9]. Results of the present study showed no effect of caffeine treatments on freeze-thawed ram spermatozoa MDA content (MDA considered as a lipid peroxidation marker). Resemble to the present study, caffeine consumption in human tended to decrease liver MDA content [4]. On the other hand, supplementing green tea (Camellia sinensis) extract in a soybean lecithin extender decreased MDA formation of post-thawing ram semen [33].

Previous studies proved that seminal plasma possesses antioxidant activity and specific enzymes which included: GPx, SOD, and catalase which they eliminate hydrogen peroxide and involve in detoxification of reactive lipids [34, 38]. Though antioxidant activities decrease during cryopreservation through high lipid peroxidation [38]. Based on our knowledge, there is no evidence regarding the effect of caffeine on antioxidant capacity of freeze-thawed animal spermatozoa. Therefore, for the first time results of the present study indicated increasing effect of caffeine in a Tris based extender on GPx and total antioxidant capacity of ram semen in a way that the highest amount was observed in 1 mM/L caffeine treatment. In an study, feeding caffeine at the level of 50 mg/kg of body weight in normal albino Wistar rats decreased oxidative stress of testes and epididymides, while increased catalase and superoxide dismutase activities [2]. Other studies with supplementing green and white tea in semen extender reported higher endogenous antioxidant capacity accompanied with lower oxidative damages of spermatozoa [16, 33]. Previous studies indicated antioxidant properties of caffeine on preventing oxidative damages and lipid peroxidation as well as improving antioxidant enzyme activities in liver, kidney, lungs, and brain cells [25, 52, 1]. In a study, it was proved that caffeine antioxidant effect against oxidative stress mediated by increasing in GSH and GSH-PX levels of mouse liver tissue [4]. Also, it was presumed that the effect of caffeine against oxidative stress may be partly mediated by antagonistic effect of caffeine on adenosine receptor (because of structural similarity between caffeine and adenosine molecule) and then, controlling the oxidative state of cells [14].

**Conclusion**

In conclusion, findings of the present study indicated that using 2mM/L concentration of caffeine in a Tris-based extender had the best increasing effect on sperm velocity parameters (measured by CASA software), while a decreasing effect on total abnormality of spermatozoa. On the other hand, adding 1 mM/L concentration of caffeine in a Tris based extender had the best improving effect on spermatozoa viability percentage and semen glutathione peroxidase and total antioxidant capacity. Accordingly, 1mM/L and 2mM/L concentrations of caffeine are advisable levels for improving Ghezel ram spermatozoa performance during cryopreservation.

**References**


