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The Effect of Coumestrol Supplementation on Ovine Semen Cryopreservation

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Running title: Effect of coumestrol on cryopreserved ram semen

Abstract

Background: Antioxidants supplementation promotes the fertilizing capacity of post-thawed ram spermatozoa.

Objectives: The study was designed to validate the effect of different levels of coumestrol as an antioxidant on post-thawing parameters of cryopreserved spermatozoa in rams.

Methods: A total of 60 ejaculates were collected from 6 sexually mature Barki rams. The accepted semen samples were extended, pooled, cooled then divided into 5 aliquots: control (tris-based egg yolk extender) without coumestrol addition, and 4 aliquots with concentrations of 0.1, 1, 10 and 100 µM of coumestrol and then subjected to cryopreservation process. The control and treated frozen semen were thawed and assessed for motility with CASA, total antioxidant capacity (TAC), malondialdehyde (MDA) level, mitochondrial activity, comet assessed DNA integrity, acrosome integrity and casein kinase 2 alpha 2 (CK2α2) gene expression.

Results: Addition of coumestrol 1µM significantly improved progressive motility. Metabolic activity was significantly higher in treated semen with 1, 10 and 100 µM coumestrol compared to control and 0.1µM coumestrol. Furthermore, acrosome integrity was significantly higher in 0.1 and 1µM of coumestrol groups. In 1, 10 and 100µM coumestrol groups TAC were significantly higher than control. Furthermore, MDA levels were significantly lower in all coumestrol groups when compared to control. Comet assay exhibited significant reduction in fragmented DNA in semen treated with coumestrol groups, especially with addition of 1 µM coumestrol. The expression of CK2α2 showed a significant fold decline in semen supplemented with 10 and 100 µM coumestrol compared to control group.

Conclusion: the addition of 1 μM coumestrol could ameliorate the deleterious impacts of cryo-damage via improving the sperm antioxidant capacity, mitochondrial activity and both acrosome a well as DNA integrities.

Keywords: Casein kinase-2 expression, Coumestrol, Cryopreservation, Mitochondrial activity, Ovine semen

Introduction

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Semen cryopreservation is one of the reproduction assisting techniques. These techniques aid in establishing and management of germplasm banks, therefore reinforcing biodiversity conservation and safe keeping the endangered species (Fickel *et al.*, 2007). Artificial insemination is the main tool for improving the genetic pool of farm animals by spreading high quality frozen semen (Abdel-Rahman *et al.*, 2000).

The cryopreserved sperm encounters chemical and physical hazards that adversely influence plasma membrane and acrosome integrity, sperm motility and livability (Kumar *et al.*, 2003). Additionally, cryopreservation alters sperm metabolic activities (Figueroa *et al.*, 2015) that resulted from thermal changes, crystallization as well as osmotic stress (Di Santo *et al.*, 2011; Fakhrildin & Hassani, 2023). Moreover, cryopreservation and subsequent thawing generate reactive oxygen species (ROS) which put spermatozoa under oxidative stress and cause DNA fragmentation (Tvrdá *et al.*, 2015). The high level of polyunsaturated fatty acids in ram spermatozoa increase the susceptibility to cryo-damage (Jumintono *et al.*, 2021) followed by impairment to sperm function and fertilization (Abdollahi *et al.*, 2021).

Many literatures suggested that protein degradation, premature protein phosphorylation and carbonylation could be the vital reasons of post-thawing sperm dysfunction (Mostek *et al.*, 2017; Wang *et al.*, 2014). Furthermore, cryopreservation interferes with the expression levels and functional condition of various proteins linked to acrosome reaction, mitochondrial activity and premature capacitation (Bogle *et al.*, 2017; Westfalewicz *et al.*, 2015).

It is known that addition of antioxidant in cooling as well as cryopreservation extender is beneficial, as it limits the oxidative stress caused by chilling or cryopreservation and thawing (Altyeb *et al.*, 2022; Mohajer et al., 2023; Varışlı *et al.*, 2021). Many phenolic plant-derivative

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compounds possess remarkable antioxidant capability to provide hydrogen atoms/ electron from their hydroxyl groups to free radicals (Mondal & Rahaman, 2020). These polyphenolic compounds are broadly distributed in the legume plants, Coumestrol is one of these compounds. It is a phytoestrogen belonging to the coumestans group (Hutabarat *et al.*, 2001). Coumestrol is well-known for various biological properties, such as antioxidant, anti-inflammatory, anti-aging, anti-adipogenesis, anti-breast cancer, inhibitor of osteoclast differentiation and estrogenic effect, with strong binding affinities for estrogen receptors α and β (Chandsawangbhuwana & Baker, 2014; Jang *et al.*, 2016; Kanno *et al.*, 2004; Koirala *et al.*, 2018; Park *et al.*, 2015; You *et al.*, 2017; Zafar *et al.*, 2017).

In this vein, the study was designed to demonstrate the influence of different concentrations of coumestrol on cryopreserved ram semen regarding CASA assessed sperm motility, acrosome integrity, serum levels of total antioxidant capacity (TAC) and malondialdehyde (MDA). Besides, mitochondrial activity, sperm DNA integrity as well as the gene expression of Casein kinase 2 alpha 2 (CK2α2) were evaluated in cryopreserved ram semen.

Materials and Methods

Animals

Six sexually mature Barki rams with a history of semen with good freezing capacity were used for the study. Rams were healthy and disease free as they were subjected to veterinarian examination routinely. Optimum housing, nutrition and lightening system were applied for rams

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during the study period. All procedures dealing with rams were approved by Suez Canal University Ethics and Animal Experimentation Committee, approval number: SCU 2023085.

Semen collection

Semen was collected twice weekly in the autumn season for five successive weeks from all the allocated rams. At the early morning, semen collection was performed with the usage of ewe as a teaser. A pre-warmed (42-45 °C) lubricated artificial vagina was used for semen collection. Acquired ejaculates were extended 1:1 directly with warmed (37°C) tris-based egg yolk extender. Extended ejaculates were evaluated for individual motility by phase contrast microscope provided with hot stage (37°C). Ejaculates with more than 70 % progressive forward motility (PFM) and 2×10^9 sperm cell per mL were accepted for further processing (Mohajer et al., 2024).

Cryopreservation of ram semen

At 37°C, accepted extended ejaculates were pooled, further extended to 200 million per mL, divided into 5 aliquots. Afterwards, the extended semen was left in a cold handling cabinet for 4 hours to be cooled to 5 °C, then these aliquots were supplemented with 0.1, 1, 10 and 100 µM of coumestrol (27885-50MG Sigma, St. Louis, MO, USA) by using 100X stock solutions of coumestrol dissolved in dimethyl sulfoxide. Half an hour equilibration period was allowed afterwards semen was packed in 0.25 mL French straws. The straws were arranged horizontally in a Styrofoam box 6cm above liquid nitrogen surface for 15 minutes. Then the straws were dipped in liquid nitrogen and stored till analysis (Barfourooshi et al., 2023).

Frozen semen analysis

Motility and kinetic CASA assessed parameters.

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Frozen straws were thawed in a 37°C water bath for 30 seconds. Progressive forward motility was assessed by the aid of computer-Assisted Sperm Analysis [(CASA), SpermVision™ software minitube, Germany]. On a pre-warmed slide (37°C), 10 µL of thawed semen sample was covered by a coverslip and examined at 400 X magnification with ovine CASA setting. At least five fields were examined as designated by **Cirit *et al.* (2013**) and Yousef et al. (2022).

Percentage of acrosome integrity

Giemsa stain was used for estimation of acrosome integrity according to Boccia *et al.* (2007). In brief, thawed semen was washed by centrifugation, smeared, air dried and then fixed for 2:4 min in 14% formalin and 86% HCL 1N. Smears were washed by water, dried and then immersed in 7% of Giemsa stock solution in distilled water overnight. Spermatozoa (200 cells) were examined for the integrity of acrosome by oil immersion lens.

Levels of total antioxidant capacity and lipid peroxidation

Spectrophotometric method was performed for evaluation the levels of TAC (Biodiagnostic, Egypt) via H_2O_2 determination in the thawed semen samples (37 C, λ= 505 nm) according to Koracevic *et al.* (2001). Furthermore, the MDA levels were used as a monitor for lipid peroxidation and as thiobarbituric acid determinant (Ohkawa *et al.*, 1979) in the semen samples. The spectrophotometer was adjusted (λ =532 nm) for MDA concentration measurement (Biodiagnostic, Egypt).

Mitochondrial activity

The MTT reduction assay was performed according to Hughes *et al.* (1996) using MTT reagent (SERVA Electrophoresis GmbH, Germany). Briefly, 100 µL of thawed semen and 10 µL MTT stock solution (5 µg MTT / 1ml PBS) were mixed in the wells of micro-plate. The

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optical densities of semen samples were detected by ELISA reader (VersaMax ELISA Microplate Reader, Molecular Devices Co., USA) at 550 nm wavelength immediately after thawing and a second time after an hour after incubation of the sample at 37°C. The difference between the 1st and 2nd readings was recorded as MTT reduction rate.

Integrity of sperm DNA

Neutral comet assay (single-cell gel electrophoresis) was implemented to detect the DNA integrity in semen samples (Bucak *et al.*, 2010; Hughes et al., 1996). Spermatozoa with extended migration of head DNA "comet" pattern represented fragmented sperm DNA. However, those with sperm head without a comet demonstrated intact sperm DNA (Sarıözkan *et al.*, 2014).

Casein kinase 2 alpha 2 (CK2α2) gene expression

For each replicate 20 straws were used for total RNA extraction. After thawing and washing of semen, total RNA was extracted by QIAzol®lysis reagent (QIAGEN, Maryland, USA), as directed by the manufacturer. Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Lithuania) was used to generate cDNA from the RNA template immediately after total RNA extraction, according to the manufacturer's instructions.

Gene expression levels for CK2α2 with β-actin as the housekeeping gene were evaluated using primers developed based on cDNA sequences deposited in GenBank (Table, 1) and Maxima SYBR Green qPCR master mix (2x) (Thermo Fisher Scientific inc., Lithuania). The

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fold difference method 2-ΔΔCt was used to determine the relative quantification of gene expression levels for target gene (Yadav *et al.*, 2013).

Statistical analysis

The results of thawed semen samples treated with different concentrations of coumestrol were statistically analyzed by using GraphPad Prism software (software version 7.0, San Diego, USA). The One-Way Analysis of Variance was conducted between the control group and the various concentrations of coumestrol regarding the post-thaw sperm criteria. Significance between groups was adjusted at probability < 0.05.

Results

CASA assessed progressive forward motility

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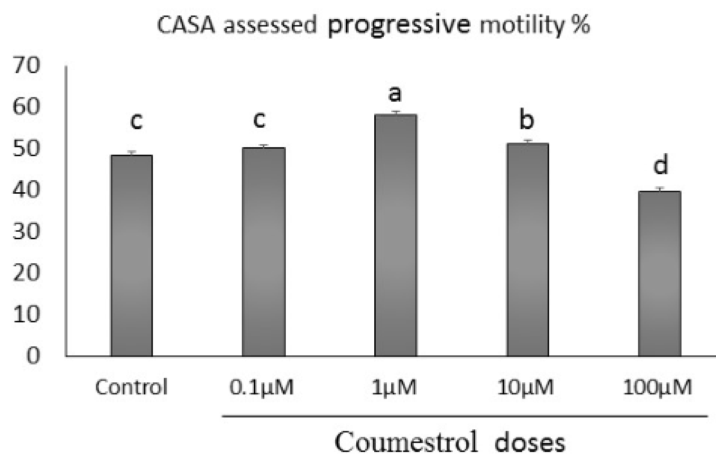

Figure 1: Post thaw CASA assessed progressive motility (%) of cryopreserved ram sperms supplemented with (0.1µM, 1µM, 10µM and 100µM) coumestrol and control. Different superscripts indicated significant variance at P<0.05 when compared with control.

There was a significant (P< 0.05) increase in CASA assessed PFM of thawed ram spermatozoa supplemented with 1 and 10 µM coumestrol as compared to control, 100 and 0.1 µM coumestrol with highest value in 1 µM group. On other hand, PFM for 100 µM coumestrol group was significantly (P<0.05) lower than all other groups (Figure, 1).

Sperm acrosome integrity

Frozen ram spermatozoa exhibited significant (P< 0.001) enhancement in acrosome integrity percentage for 0.1 and 1 µM coumestrol groups compared with control with highest integrity in 1 µM group. However, 100 µM concentration exhibited significantly (P< 0.05) reduced acrosomal integrity than control (Table, 2).

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Total antioxidant capacity and malondialdehyde levels

Table (3) revealed that coumestrol 1 and 10µM produced a significant (P< 0.01) improvement in the TAC levels when compared to 0.1 and 100µM coumestrol. Lipid peroxidation demonstrated significant (*P*< 0.01) reduction of MDA levels in all coumestrol supplemented groups as compared to the control group with lowest value in the 1 µM group.

Mitochondrial activity

Regarding MTT reduction, 1, 10 and 100 µM coumestrol groups had significantly (P< 0.01) higher MTT reduction rate than control with the lowest values in 10µM group. Moreover, both the control and 0.1 µM coumestrol had non-significant difference (Table, 3).

Sperm DNA integrity

As illustrated in Table (4), the comet assay of ram sperm DNA revealed that the addition of coumestrol to cryopreserved ram semen significantly (*P* <0.05) diminished the comet % in all groups. Additionally, 1 and 10µM coumestrol had (*P* <0.05) had the lowest comet% as when compared with 0.1 and 100µM coumestrol groups.

Casein kinase 2 alpha 2 gene expression

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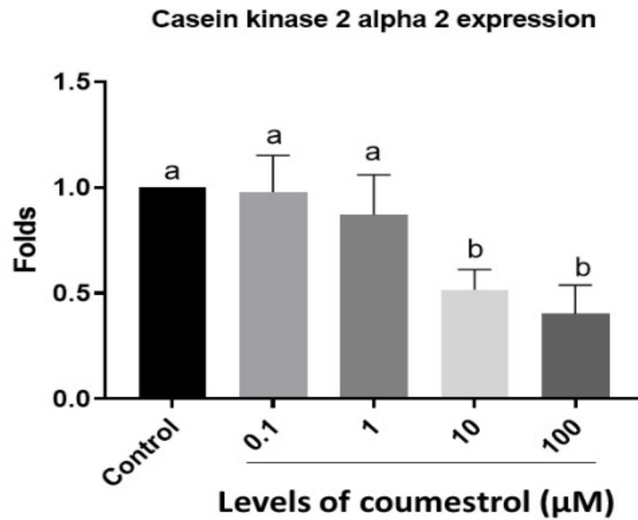

Figure 2: Expression of Casein kinase 2 alpha 2 gene in extended ram semen supplemented with 0, 0.1µM, 1µM, 10µM and 100µM coumestrol. Different superscripts indicated significant variance at P<0.05 when compared with control.

As shown in Figure 2, addition of 0.1 and 1 µM coumestrol to cryopreserved ram's semen extender resulted in non-significant decrease in CK2α2 mRNA fold change as compared to control. On the other side, the addition of 10 and 100µM coumestrol to extender resulted in significant (*P* <0.05) downregulation of CK2α2 gene expression than control and other coumestrol groups.

Discussion

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Phytoestrogens were used to improve fertility of cryopreserved ram semen (Elsayed *et al.*, 2019). However, there are inconstancies regarding the effect of phytoestrogens on semen. A study performed by Mitchell *et al.* (2001) confirmed that human consumption of phytoestrogen-rich diets showed no alterations on semen criteria regarding volume, sperm concentration, motility as well as morphology. On the other side, phytoestrogen reduced sperm functionality via lowered sperm motility, increased membrane fluidity, enhanced DNA fragmentation and promoted lipid peroxidation of ram semen (Pool *et al.*, 2021). However, Elsayed et al. (2019) reported pronounced improvement when ram cryopreservation extender was supplemented with genistein. Therefore, the present study was designed to investigate the optimum coumestrol concentration(s) on rams' sperms functionality of cryopreserved semen.

Freezing and thawing processes expose the spermatozoa to sever temperature changes, ice crystal formation and subsequent osmotic stress, which leads to oxidative, biochemical and structural damage in spermatozoa (Gürler *et al.*, 2016). Levels of natural semen antioxidants decreases after dilution and during cryopreservation due to excessive generation of ROS (Kumar *et al.*, 2011). This oxidative stress induces lipid peroxides and MDA formation, which disrupt sperm membrane integrity, reduce sperm motility, increase DNA fragmentation, damage mitochondria, reduce sperm viability and promote apoptosis (Gürler et al., 2016; Sikka, 2001). Therefore, it is essential to maintain antioxidants defenses during freezing and thawing (Reddy *et al.*, 2018).

In our study, the addition of 1 μM coumestrol to cryopreservation extender exhibited best overall results. It significantly increased post thaw PFM, acrosome intactness. The PFM motility is required for spermatozoa to be able to penetrate cervical mucus to reach the uterine tube and

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penetrate oocyte's zona pellucida. Physiologically functional spermatozoa are highly motile, whereas decreased sperm metabolism or failing organelles cause low motility. These low motility spermatozoa have lower probability to undergo capacitation, to reach the oocyte or to fertilize the oocyte (Aitken *et al.*, 2012; Martinez-Pastor *et al.*, 2009). Whereas 100µM coumestrol significantly decreased PFM compared to control denoting negative influence of such concentration on sperm motility. Acrosomal integrity has been confirmed as an important indicator of fertility of cryopreserved semen in bulls (Bernecic *et al.*, 2021) and ram (Vozaf *et al.*, 2022). Our study revealed that 0.1 and 1 µM coumestrol significantly increased acrosome integrity that denotes the positive influence of coumestrol on sperm fertility. However, 100µM coumestrol decreased acrosomal integrity as compared to control denoting negative influence of such concentration on sperm acrosome.

Coumestrol is considered the most potent phytoestrogens with an affinity for mammalian estrogen. It binds to estrogen receptors α and β (ERα and ERβ) with relatively high affinity (Kuiper *et al.*, 1997; Zsarnovszky & Belcher, 2001). Ovine spermatozoa have both ERα and ERβ. The ERβ decreases after capacitation and almost disappears after the acrosome reaction (Gimeno-Martos *et al.*, 2017). In goat semen cryopreservation, the group supplemented with 3µM estradiol had the highest PGF when compared to 5µM estradiol and control groups (Mesbah *et al.*, 2022). However, incubation of ovine semen at 39 °C for three hours with estradiol or its agonist resveratrol significantly caused hyperactivation of the spermatozoa, diminish PFM and acrosome integrity (Gimeno-Martos et al., 2017). So, the adverse impact of 100µM coumestrol regarding PFM and acrosome integrity

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can be attributed to the overstimulation of estrogen receptors causing sperm hyperactivation and acrosome reaction.

Present results revealed significant promotion of TAC while reduction in MDA sperm contents especially at 1 and 10 µM coumestrol concentrations. Coumestrol is characterized by the presence of a coumarin and benzofuran fused ring system and two hydroxyl groups, which are responsible for both estrogenic and antioxidant capacities (Chandsawangbhuwana & Baker, 2014; Xi & Liu, 2014). Hydroxyl groups in the A and B-rings act as free radicals' scavengers due to their ability to donate H atoms/electrons to the free radicals (Dabrowska & Wiczkowski, 2017). Therefore, they can relief cells from oxidative stress (Diplock, 1994). Coumestrol had more powerful antioxidant capability than genistein. (Mitchell *et al.*, 1998). Using Electron Spin Resonance Spectroscopy System by the later authors revealed that coumestrol quenched 0.102 radicals per molecule while genistein quenched less than 0.02 radicals per molecule. In ferric-reducing ability of plasma assay, coumestrol had more than double of genistein's ferric reducing ability but roughly half Trolox, and vitamin C abilities. Low coumestrol concentration 0.1µM was insufficient to produce a pronounced antioxidant potential in the present study, while 100µM coumestrol may exceed the physiological concentration that can produce the favorable antioxidant effect since its TAC level was significantly lower than 10µM coumestrol.

The MTT is valuable for sperm cell viability evaluation (Buranaamnuay, 2021; Momeni *et al.*, 2020). Both ERα and ERβ are present in the mitochondria of various cell types and tissues; independent stimulation of either these receptors enhanced sperm mitochondrial activity (Psarra & Sekeris, 2008). Irwin *et al.* (2012) reported that coumestrol administration enhanced brain mitochondrial energy-transducing capacity through balanced respiration and reduced oxidative

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damage**.** Coumestrol was reported as an effective inducer of mitochondrial biogenesis. It improved ovariectomized rats' brain and liver mitochondrial function by increasing the respiratory control ratio and reducing oxidative stress (Moreira *et al.*, 2017). Furthermore, differentiated myocytes cell line mitochondria had elevated cellular ATP levels, glucose uptake NAD+/NADH ratio and sirtuin1 activity when incubated with coumestrol (Seo *et al.*, 2014). Current study augments previous results whereas it denotes the positive influence of coumestrol on sperm mitochondrial activity.

DNA fragmentation reduces fertilization capacity either due to arrest of early embryo development and/or fertilization prevention (Gallo *et al.*, 2018). Sperm DNA integrity is considered an important marker of sperm quality. It is paramount to protect DNA from freeze–thaw-induced damage to achieve better cryopreservation (Isaac *et al.*, 2017). The present results revealed the most significant reduction in comet % was in 1 and 10 µM coumestrol, which that harmonized with the elevated TAC and mitochondrial activity with reduced MDA in such groups. The later concentrations of coumestrol seems to be optimum for sperms to have estrogenic antioxidant potential that reduces free radicals attack to sperm DNA (Noegroho *et al.*, 2022).

The present results denoted that CK2α2 mRNA expression for 0.1 and 1µM coumestrol was non-significantly altered than control. However, 10 and 100 µM showed significant reduction in CK2α2 mRNA expression fold change with the lowest values in 100 µM concentration. Coumestrol is a potent and selective CK2α2 protein kinase inhibitor (Baier & Szyszka, 2020)**.** The CK2α2 molecule is a serine/threonine-selective kinase. It regulates the

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function of several proteins such as transcription factors, growth factor receptors and cytoskeletal proteins. Furthermore, CK2α2 is anti-apoptotic protein, it phosphorylates Bid, which then blocks caspase-8 activation, thereby preventing apoptosis (Mannowetz *et al.*, 2010). The freezing and thawing processes for ram sperm significantly decrease the amount CK2α2 protein, DNA integrity and acrosome integrity. Moreover, they induce CK2α2 mRNA degradation. The amount of CK2α2 mRNA inverse significantly correlated with DNA and acrosome integrity (He *et al.*, 2017). The concentrations 0.1 and 1µM of coumestrol did not significantly alter CK2α2 expression. So, these concentrations had no deleterious effects. On the other hand, 100µM group produced significant suppression of CK2α2 mRNA expression, which may be reflected in the lower values of acrosome integrity and DNA integrity.

Our study demonstrated that 1µM coumestrol seems to be physiologically optimum to influence antioxidant capability of rams' semen that further enhances the PFM and sperm integrity and mitochondrial activity. Besides, its possible modest activation to sperms' ERα and ERβ that encounter several criteria of sperms' motility and integrity than the other coumestrol doses. In addition to, maintenance of CK2α2 expression non altered therefore acrosome and DNA integrity were not altered.

Conclusions

Supplementation of cryopreservation extender with 1µM coumestrol improved post thaw parameters through modulation of antioxidants, acrosomal integrity, mitochondrial activity and DNA integrity without altering CK2α2 expression. So, the use of 1µM coumestrol in cryopreservation extender could enhance the overall quality of cryopreserved ram spermatozoa.

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Declaration of competing interest

The authors declare no conflict of interest.

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