Original Article

Effect of Coumestrol Supplementation on Ovine Semen Cryopreservation

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ABSTRACT

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

Objectives: This 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 six sexually mature Barki rams. The accepted semen samples were extended, pooled, cooled, and then divided into five aliquots: A control group (tris-based egg yolk extender) without coumestrol addition, and 4 aliquots with concentrations of 0.1, 1, 10, and 100 µM of coumestrol. These aliquots were then subjected to the cryopreservation process. The control and treated frozen semen were thawed and assessed for motility using computer-assisted sperm analysis (CASA), total antioxidant capacity (TAC), malondialdehyde (MDA) levels, mitochondrial activity, comet assay for DNA integrity, acrosome integrity, and casein kinase 2 alpha 2 (*CK2α2*) gene expression.

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

Conclusion: The addition of 1 µM of coumestrol could ameliorate the deleterious impacts of cryo-damage by improving the sperm antioxidant capacity, mitochondrial activity, and both acrosome and DNA integrity.

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

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Introduction

Semen cryopreservation is one of the reproduction assistance techniques. These techniques aid in establishing and managing germplasm banks, thereby reinforcing biodiversity conservation and safeguarding 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).

Cryopreserved sperm encounters chemical and physical hazards that adversely influence plasma membrane and acrosome integrity, as well as sperm motility and livability (Kumar et al., 2003). Additionally, cryopreservation alters sperm metabolic activities (Figueroa et al., 2015) that result from thermal changes, crystallization, and osmotic stress (Di Santo et al., 2011; Fakhrildin & Hassani, 2023). Moreover, cryopreservation and subsequent thawing generate reactive oxygen species (ROS), which expose spermatozoa to oxidative stress and cause DNA fragmentation (Tvrdá et al., 2015). The high levels of polyunsaturated fatty acids in ram spermatozoa increase the susceptibility to cryodamage (Jumintono et al., 2021), leading to impairments in sperm function and fertilization (Abdollahi et al., 2021). Cryopreserved sperm encounters chemical and physical hazards that adversely affect.

Protein degradation, premature protein phosphorylation, and carbonylation can cause 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 the addition of antioxidants to the cryopreservation extender is beneficial, as it limits the oxidative stress caused by cryopreservation and thawing (Altyeb et al., 2022; Varışlı et al., 2021). Many phenolic plant-derived compounds possess remarkable antioxidant capabilities, providing hydrogen atoms or electrons from their hydroxyl groups to free radicals (Mondal & Rahaman, 2020). These polyphenolic compounds are broadly distributed in the legume plants, and 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 effects, inhibition of osteoclast differentiation, and estrogenic effects, 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 context, this study was designed to demonstrate the influence of different concentrations of coumestrol on cryopreserved ram semen regarding computer-assisted sperm analysis (CASA)-assessed sperm motility, acrosome integrity, total antioxidant capacity (TAC), and serum malondialdehyde (MDA) levels. 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. Rams were healthy and disease-free, as they underwent routine veterinary examinations. Optimum housing, nutrition and lightening system were applied for rams during the study period.

Semen collection

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

Cryopreservation of ram semen

At 37 ˚C, the accepted extended ejaculates were pooled, further extended to 200 million per mL, and divided into five aliquots. Afterward, 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) using 100x stock solutions of coumestrol dissolved in dimethyl sulfox-

ide. Following a half-hour equilibration period, the semen was packed into 0.25 mL French straws. The straws were arranged horizontally in a Styrofoam box, 6 cm above the liquid nitrogen surface, for 15 minutes. Then, the straws were dipped in liquid nitrogen and stored until analysis.

Frozen semen analysis

Motility and kinetic CASA-assessed parameters

Frozen straws were thawed in a 37 ˚C water bath for 30 seconds. Progressive forward motility was assessed through computer-assisted sperm analysis (CASA) with SpermVision™ software (Minitube, Germany). On a pre-warmed slide (37 ˚C), 10 µL of the thawed semen sample was covered with a coverslip and examined at 400X magnification with the ovine CASA settings. At least five fields were examined as designated by Cirit et al. (2013).

Percentage of acrosome integrity

Giemsa stain was used to estimate 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 a solution of 14% formalin and 86% 1N HCl. Smears were washed with 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 an oil immersion lens.

Levels of TAC and lipid peroxidation

The spectrophotometric method was performed to evaluate 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. (Koracevic et al., 2001). Furthermore, MDA levels were used as a marker for lipid peroxidation and as a 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 (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed (Hughes et al., 1996) using an MTT reagent (SERVA Electrophoresis GmbH, Germany). Briefly, 100 µL of thawed semen and 10 µL of MTT stock solution (5 µg MTT / 1 mL PBS) were mixed in the wells of a microplate. The optical densities of semen samples were detected by an ELISA reader (VersaMax ELISA Microplate Reader, Molecular Devices Co., USA) at 550 nm immediately after thawing and an hour after incubation of the sample at 37 ˚C. The difference between the first and second readings was recorded as the MTT reduction rate.d as the MTT reduction rate.

The integrity of sperm DNA

The 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 in a "comet" pattern indicated fragmented sperm DNA. However, those with sperm heads that did not display a comet demonstrated intact sperm DNA (Sarıözkan et al., 2014).

CK2α2 gene expression

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

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 fold difference method, $2^{-\Delta\Delta Ct}$, was used to determine the relative quantification of gene expression levels for the target gene (Yadav et al., 2013).

Statistical analysis

The results of thawed semen samples treated with different concentrations of coumestrol were statistically analyzed using GraphPad Prism software, version 7 (San Diego, USA). The one-way analysis of variance was conducted to compare the control group with various concentrations of coumestrol regarding the post-thaw sperm criteria. Significance between groups was set at a probability of $P<0.05$.

Table 1. Primers of sheep *CK2α2* expression and beta actin genes for real time PCR

Gene	Sequence (5'- 3')	Annealing Temperature	Product length (bp)	References
Sheep *CK2α2*	F: 5'- TCTGGAGAACCTTCGTGGTG -3' R: 5'- CCTGTGCATGATTCCCTTGC -3'	59 °C	214	(Gene ID: 101122459) Accession:XM_027977894.3
Sheep *Beta actin*	F: 5'-ACTGGGACGACATGGAGAAG-3' R: 5'-GGGGTGTTGAAGGTCTCAA-3'	59 °C	156	Lengi & Corl, 2008

Results

Progressive forward motility

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

Sperm acrosome integrity

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

TAC and MDA levels

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

Mitochondrial activity

Regarding MTT reduction, the 1, 10, and 100 µM coumestrol groups had significantly (P<0.01) higher MTT reduction rates than the control, with the lowest values in the 10 µM group. Moreover, both the control and 0.1 µM coumestrol groups showed no 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 percentage of comet formation in all groups. Additionally, 1 and 10 µM coumestrol had the lowest percentage of comet formation (P<0.05) when compared with the 0.1 and 100 µM coumestrol groups.

CK2α2 gene expression

As shown in Figure 2, the addition of 0.1 and 1 µM coumestrol to the cryopreserved ram semen extender resulted in a non-significant decrease in CK2α2 mRNA fold change compared to the controls. On the other hand, the addition of 10 and 100 µM coumestrol to the extender resulted in a significant (P<0.05) downregulation of *CK2α2*

Table 2. Post thaw acrosome integrity of cryopreserved ram sperms supplemented with (0.1 µM, 1 µM, 10 µM and 100 µM) coumestrols and control

Group	Mean±SD
	Acrosome Integrity (%)
Control	64.00±1.70^{a}
0.1 µM coumestrol	70.60±1.36^{b}
1 µM coumestrol	74.80±1.36^{c}
10 µM coumestrol	64.20±0.37^{a}
100 µM coumestrol	58.20±0.58^{d}

Notes: Different superscripts in the same column are significantly different P<0.05.

Table 3. TAC, MDA levels and MTT reduction rate of cryopreserved ram sperms supplemented with (0.1 µM, 1 µM, 10 µM and 100 µM) coumestrols and control

Group	Mean±SD		
	TAC (mmol/mL)	MDA (nmol/mL)	MTT Reduction Rate
Control	1.86±0.01[a]	13.47±0.35[a]	0.42±0.07[a]
0.1 µM coumestrol	1.93±0.02[a]	10.51±0.45[b]	0.45±0.07[ab]
1 µM coumestrol	2.01±0.01[b]	7.77±0.32[c]	0.62±0.08[b]
10 µM coumestrol	2.05±0.02[b]	10.59±0.52[b]	0.86±0.04[c]
100 µM coumestrol	1.90±0.05[a]	10.64±0.39[b]	0.63±0.07[b]

Notes: Different superscripts in the same column are significantly different P<0.05.

gene expression compared to the control and the other coumestrol groups.

Discussion

Phytoestrogens have been used to improve the fertility of cryopreserved ram semen (Elsayed et al., 2019). However, there are inconsistencies regarding the effect of phytoestrogens on semen. Mitchell et al. (2001) confirmed that human consumption of phytoestrogen-rich diets showed no alterations in semen criteria regarding volume, sperm concentration, motility, or morphology. On the other hand, phytoestrogen reduces sperm functionality by lowering sperm motility, increasing membrane fluidity, enhancing DNA fragmentation, and promoting lipid peroxidation in ram semen (Pool et al., 2021). However, Elsayed et al. (2019) reported a pronounced improvement when the ram cryopreservation extender was supplemented with genistein. Therefore, the present study was designed to investigate the optimum coumestrol concentration(s) for enhancing the functionality of ram sperm in cryopreserved semen.

Freezing and thawing processes expose the spermatozoa to severe 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 decrease 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 antioxidant defenses during freezing and thawing (Reddy et al., 2018).

In our study, the addition of 1 µM coumestrol to the cryopreservation extender exhibited the best overall results. It significantly increased post-thaw PFM and

Table 4. Sperm DNA integrity assessed by neutral comet assay of cryopreserved ram sperms supplemented with (0.1 µM, 1 µM, 10 µM and 100 µM) coumestrols and control

Group	Mean±SD		
	Comet (%)	Tail Length (Px)	DNA Tail (%)
Control	10.52±0.22[a]	6.54±0.59[a]	4.05±0.86[a]
0.1 µM coumestrol	7.80±1.07[b]	6.51±0.51[a]	3.49±0.46[a]
1 µM coumestrol	4.40±0.40[c]	4.94±0.42[a]	4.03±0.62[a]
10 µM coumestrol	5.00±0.71[c]	6.06±0.23[a]	4.20±0.41[a]
100 µM coumestrol	6.90±0.43[b]	6.43±0.42[a]	4.56±0.49[a]

Notes: Different superscripts in the same column are significantly different P<0.05.

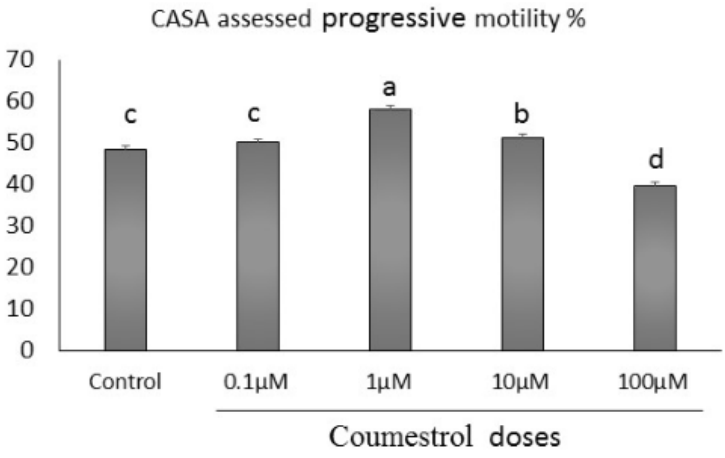

Figure 1. Post thaw CASA assessed progressive motility (%) of cryopreserved ram sperms supplemented with (0.1 µM, 1 µM. 10 µM and 100 uM) coumestrols and control

Notes: Different superscripts indicate significant differences compared to the control (P<0.05).

acrosome intactness. PFM is essential for spermatozoa to penetrate cervical mucus, reach the uterine tube, and penetrate the oocyte's zona pellucida. Physiologically functional spermatozoa are highly motile, whereas decreased sperm metabolism or failing organelles cause low motility. Spermatozoa with low motility have a lower probability of undergoing capacitation, reaching the oocyte, or fertilizing the oocyte (Aitken et al., 2012; Martinez-Pastor et al., 2009). In contrast, 100 µM coumestrol significantly decreased PFM compared to the control, indicating a negative influence of this concentration on sperm motility. Acrosomal integrity has been confirmed as an important indicator of fertility in cryopreserved semen from bulls (Bernecic et al., 2021) and rams (Vozaf et al., 2022). Our study revealed that 0.1 and 1 µM coumestrol significantly increased acrosome integrity, indicating a positive influence of coumestrol on sperm fertility. However, 100 µM coumestrol decreased acrosomal integrity compared to the controls, suggesting a negative influence of this concentration on sperm acrosome integrity.

Coumestrol is considered the most potent phytoestrogen, 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 expression of ERβ decreases

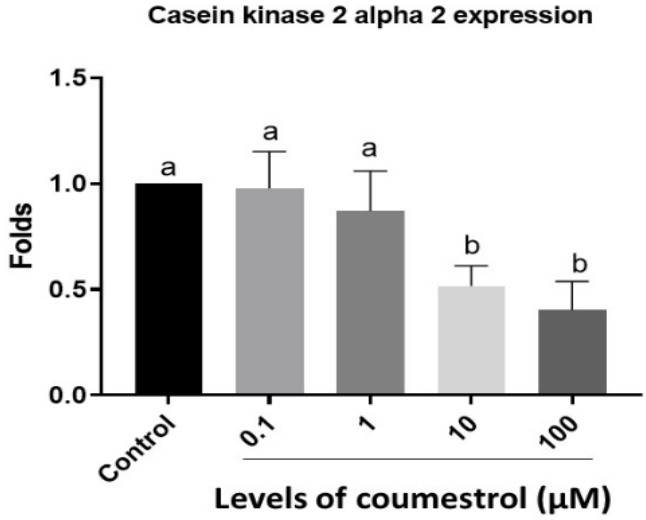

Figure 2. Expression of *CK2a2* gene in extended ram semen supplemented with 0, 0.1 µM, I µM. 10 µM and 100 µM coumestrols

Notes: Different superscripts indicate significant differences compared to the control (P<0.05).

after capacitation and almost disappears following the acrosome reaction (Gimeno-Martos et al., 2017). In goat semen cryopreservation, the group supplemented with 3µM estradiol had the highest PFM compared to the 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, diminishing PFM and acrosome integrity (Gimeno-Martos et al., 2017). Thus, the adverse impact of 100 µM coumestrol regarding PFM and acrosome integrity can be attributed to the overstimulation of estrogen receptors, causing sperm hyperactivation and acrosome reaction.

The present results revealed a significant promotion of TAC alongside a reduction in MDA sperm content, especially at 1 and 10 µM coumestrol concentrations. Coumestrol is characterized by the presence of a coumarin and benzofuran fused ring system, along with two hydroxyl groups, which are responsible for both its estrogenic and antioxidant capacities (Chandsawangbhuwana & Baker, 2014; Xi & Liu, 2014). The hydroxyl groups in the A and B rings act as free radical scavengers due to their ability to donate hydrogen atoms or electrons to free radicals (Dabrowska & Wiczkowski, 2017). Therefore, they can relieve cells from oxidative stress (Diplock, 1994). Coumestrol exhibits more powerful antioxidant capability than genistein (Mitchell et al., 1998). Using an electron spin resonance spectroscopy system, the later authors revealed that coumestrol quenched 0.102 radicals per molecule while genistein quenched less than 0.02 radicals per molecule. In the ferric-reducing ability of plasma assay, coumestrol had more than double the ferric-reducing ability of genistein, but roughly half that of Trolox and vitamin C. The low coumestrol concentration of 0.1 µM was insufficient to produce a pronounced antioxidant potential in the present study, while 100 µM coumestrol may exceed the physiological concentration needed to produce a favorable antioxidant effect, as its TAC level was significantly lower than that of 10 µM coumestrol.

The MTT assay 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 of these receptors enhances 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 damage. Coumestrol has been 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 myocyte cell line mitochondria had elevated cellular ATP levels, glucose uptake, NAD+/NADH ratio, and sirtuin 1 activity when incubated with coumestrol (Seo et al., 2014). The current study augments previous results, indicating the positive influence of coumestrol on sperm mitochondrial activity.

DNA fragmentation reduces fertilization capacity either by arresting early embryo development or by preventing fertilization (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 percentage occurred at 1 and 10 µM coumestrol, which correlated with the elevated TAC and mitochondrial activity, along with reduced MDA levels in these groups. These concentrations of coumestrol appear to be optimal for sperm, providing estrogenic antioxidant potential that reduces free radical attacks on sperm DNA (Noegroho et al., 2022).

CK2α2 mRNA expression at 0.1 and 1 µM coumestrol was not significantly altered compared to the control. However, 10 and 100 µM coumestrol showed a significant reduction in *CK2α2* mRNA expression fold change, with the lowest values observed at the 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 that regulates the function of several proteins, such as transcription factors, growth factor receptors, and cytoskeletal proteins. Furthermore, CK2α2 is an anti-apoptotic protein; it phosphorylates Bid, which blocks caspase-8 activation, thereby preventing apoptosis (Mannowetz et al., 2010). The freezing and thawing processes for ram sperm significantly decrease the amount of CK2α2 protein, as well as DNA and acrosome integrity. Moreover, these processes induce *CK2α2* mRNA degradation. The amount of *CK2α2* mRNA is significantly inversely correlated with DNA and acrosome integrity (He et al., 2017). The concentrations of 0.1 and 1 µM of coumestrol did not significantly alter *CK2α2* expression, indicating that these concentrations had no deleterious effects. On the other hand, the 100 µM group showed significant sup-

pression of CK2α2 mRNA expression, which may be reflected in the lower values of acrosome integrity and DNA integrity.

Our study demonstrated that 1 µM coumestrol seems to be physiologically optimal for influencing the antioxidant capability of ram semen, which further enhances PFM, sperm integrity, and mitochondrial activity. Besides, its potential modest activation of sperm ERα and ERβ may meet several criteria for sperm motility and integrity better than the other coumestrol doses. Furthermore, the maintenance of *CK2α2* expression was not altered, and therefore, acrosome and DNA integrity were also preserved.

Conclusions

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

Ethical Considerations

Compliance with ethical guidelines

All procedures dealing with rams were approved by the Ethics and Animal Experimentation Committee of Suez Canal University, Ismailia, Egypt (SCU 2023085).

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Authors' contributions

Investigation: Doaa H. Elsayed, and Laila E. Kortam; Formal analysis: Laila E. Kortam; Data curation, and writing the original draft: Doaa H. Elsayed; Methodology, review, and editing: Ahmed Monir, and Heba M.A. Abdelrazek; Supervision: Heba M.A. Abdelrazek.

Conflict of interest

The authors declared no conflicts of interest.

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