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F Avdatek, SB Berk

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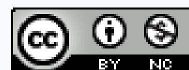

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Research article
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Effects of Proanthocyanidin on Freezability of Ram Sperm

Fatih Avdatek,* Saltuk Buğra Berk

Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

ABSTRACT: This study aimed to investigate the effect of different concentrations of proanthocyanidin (PA) added to the semen diluent of Sönmez rams on semen quality. Ejaculates were gathered from three rams once a week with an artificial vagina. The ejaculates were separated into five groups with diluents without (control, C) and containing (10, 25, 50, and 100 µg/ml) antioxidants. Samples were equilibrated for 3 hours at 5 ^{0}C and then frozen in liquid nitrogen vapor. The frozen straws were thawed at 37°C and evaluated in terms of sperm parameters, oxidative stress markers, and DNA damage. The increase in motility and the decrease in the head, tail, and total abnormal spermatozoa rates in the 10 µg/ml PA group were statistically significant ($p<0.05$) compared to the C. The increase in H+/E− (H+: host with intact membrane integrity; E−: viable spermatozoon) was statistically significant ($p < 0.05$) in the 10 µg/mL PA group compared to the C group. The decrease in tail length, tail DNA in the groups with 10, 25 µg/ml PA added, and the decrease in tail moment in all antioxidant-added groups was statistically important ($p<0.001$) compared to the C. The increase in TOS was statistically significant ($p<0.05$) in the groups with 10, 25, and 100 µg/ml PA added compared to the C. In conclusion, it was observed that the 10 µg/ml dose of PA added to the semen provided the best protection on semen quality compared to other groups in storing ram semen.

Keyword: Ram, Sperm, Proanthocyanidin, Antioxidant, Cryopreservation.

Correspondence author:
F. Avdatek,
Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey
E-mail address: favdatek@aku.edu.tr

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INTRODUCTION

Artificial insemination with frozen semen is a crucial biotechnology used to enhance the genetics of farm animals. It promotes the rapid and widespread dissemination of desired traits in males with high fertility potential. Sperm freezing allows for the conservation of genetic material and the efficient utilization of spermatozoa through artificial insemination, facilitating the selection of the best donor for optimal results (Ruane, 2000). The procedure involves various stages, including sperm dilution, incubation, cooling, freezing, and thawing, requiring a high adaptability (Medeiros *et al.,* 2002). Spermatozoa may experience varying degrees of ultrastructural, biochemical, and functional damage, resulting in reduced motility, membrane integrity, and fertilization capacity. The damage is believed to be caused by extracellular and intracellular ice crystal formation in sperm cells. Ram sperm, in comparison to other species, has a lower cholesterol-phospholipid ratio in the cell membrane, making them more sensitive to cold shock (Salamon and Maxwell, 1995). Temperature fluctuations during cooling induce stress on sperm cell membranes, leading to phase transitions in lipids and changes in spermatozoa membrane functionality. The damage during the freezing of ram sperm is attributed to increased oxidative stress due to ice crystal formation, leading to lipid peroxidation. Notably, increased oxidative stress affects not only lipids but also proteins within the spermatozoa (Türk, 2015). Substances that prevent oxidation caused by free radicals are known as antioxidants and can capture and stabilize free radicals (Ellıot, 1999). The addition of antioxidants to sperm diluents can reduce oxidative stress and improve the quality of frozen sperm during storage, as well as prevent DNA damage and scavenge reactive oxygen species (ROS) produced by leukocytes (Qamar et al., 2022).

Proanthocyanidins (PA) are active compounds derived from Vitis vinifera seeds with protective effects against oxidative stress caused by free radicals and reactive oxygen species (Hala *et al.,* 2010). Over the past decade, plant- and food-derived PA has also attracted the attention of the food industry and public health organizations for their ability to prevent chronic diseases (Nie and Stürzenbaum, 2019). PA has antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilatory effects. Additionally, they have been shown to inhibit lipid peroxidation, platelet aggregation, capillary permeability, and fragility. Structurally, PA contains conjugated hydroxyl groups with multiple phenolic hydroxyl groups, enabling them to act as potent free radical scavengers that rapidly penetrate the body and compete with free radicals, protecting the organism against lipid oxidation. Proanthocyanidin added to the extender during the short-term storage of ram sperm was found to have beneficial effects on spermatozoon motility and membrane integrity (Avdatek et al., 2020). According to Lü Songjie et al. (2019), the addition of 10 μg/mL PC during the freezing of Hu sheep semen reduced oxidative damage and improved sperm quality after thawing. However, when the concentration was increased to 40 μg/mL, PC exhibited toxic effects on Hu sheep sperm. Mu et al. (2020) concluded that dietary supplementation with grape seed procyanidins enhance growth and improves the antioxidant status of the testes and epididymis in rams, ultimately leading to improved semen quality. In this study, the optimal supplementation level was determined to be 20 mg/kg BW. Proanthocyanidin added to the extender during the short-term storage of ram sperm was found to have beneficial effects on spermatozoon motility and membrane integrity (Avdatek et al., 2020). Additionally, it has been shown that PA inhibits the activity of xanthine oxidase, which is a free radical generator. This suggests that PA has an antioxidant effect by positively influencing the expression of enzymatic antioxidants and, at the same time, preventing the formation of free radicals (Fine, 2000; Bagchi *et al.,* 2000). Despite the extensive research on the protective effects of antioxidants in sperm preservation, there is still a lack of comprehensive studies examining the optimal concentration of proanthocyanidins (PA) in extenders and their precise impact on frozen ram sperm quality. This study aims to address this gap by evaluating the effectiveness of different PA concentrations in improving sperm motility, viability, membrane integrity, oxidative stress parameters, and DNA integrity, providing novel insights into the potential use of PA in sperm cryopreservation. This research aims to assess the impact of varying concentrations of PA incorporated into the extender on the motility, rates of abnormal and viable sperm, membrane integrity, parameters related to oxidative stress, and DNA damage in frozen ram sperm.

MATERIAL AND METHOD

Semen Collection and Freezing

In our research, semen samples were obtained from three rams using an artificial vagina. Semen collection was performed with six repetitions for each group during the breeding season. A ram semen

extender based on Tris was utilized. Following a preliminary study to determine working doses based on the molecular weight of PA, semen samples were **separated i**nto five groups, and each group was separately supplemented with 0, 10, 25, 50, and 100 µg/mL of PA (respectively named as control (C), PA10, PA25, PA50, PA100). Subsequently, the samples, drawn into French straws (at a concentration of $15x10^6$), were stored at 4°C until they reached thermal equilibrium (2 hours). After ensuring optimal cooling conditions, all samples were frozen in liquid nitrogen vapor for 12 minutes. The straws were stored in liquid nitrogen (-196°C) until examination day. The frozen straws were thawed at 37°C for 20 seconds for evaluation. The research obtained ethical approval from the Animal Experiments Local Ethical Committee of Afyon Kocatepe University (AKÜHADYEK) under reference numbers 49533702/120 and AKÜHADYEK-97-21.

Evaluation of Semen Parameters

Assessment of Sperm Motility

To assess sperm motility, a drop of semen and isotonic diluent were mixed on a heated stage (37°C) phase-contrast microscope. Images were captured using a 100x objective, and evaluations were performed with 200x and 400x objectives. Spermatozoa showing linear motion in a single direction were identified, and the percentage of these spermatozoa relative to all spermatozoa in the same microscopic field was recorded. All motility evaluations were made subjectively by examining at least five different fields. Spermatozoa showing linear motion in a single direction were identified, and the percentage of these spermatozoa relative to all spermatozoa in the same microscopic field was recorded (Avdatek and Gündoğan, 2018).

Determination of Abnormal Spermatozoa Ratio

The ratio of abnormal spermatozoa was determined using the Giemsa staining method. Ten microliters of semen were placed on a slide, fixed with alcohol after making a smear, and left to dry. After a 45-minute incubation, the slides were washed, and 400 spermatozoa were counted under a microscope at 40x magnification to determine the percentage of abnormal spermatozoa in terms of head, midpiece, tail, and total anomalies (Watson, 1975).

Hypo-Osmotic Eosin Staining Test (HE Test) Assessment

Membrane integrity and the live-dead spermatozoa ratio were evaluated through the HE test. In Eppendorf tubes, sequential addition of 1 milliliter of a 100 mOsm solution, 10 µl of semen, and 20 µl of eosin dye took place. Subsequently, the tubes were incubated at 37°C for 30 minutes. Following incubation, a small mixture sample was placed on a microscope slide, smears were created, and the slides were rapidly air-dried for analysis. Subsequently, under 40x magnification, 200 spermatozoa were classified into four categories based on the presence or absence of dye in the head and kinking or swelling in the tail: H+/E- (swollen tail, no dye in the head), H-/E- (unswollen tail, no dye in the head), H+/E+ (swollen tail, dye in the head), and H-/E+ (unswollen tail, dye in the head) (Avdatek et al., 2018).

Assessment of DNA Damage

Following a 30-second thawing period at 37°C, the samples were subjected to a cold wash using PBS devoid of calcium and magnesium. Subsequently, they were centrifuged at 800x g for 10 minutes at +4°C. This process was repeated at least twice, and the samples were eventually diluted to have $20x10^6$ cells/ml. Initially, clean slides were coated with a 0.75% low melting point agarose (LMA) as the first layer, and this layer was left to air dry at room temperature. Subsequently, 10 µl of diluted semen and 90 µl of 1% LMA were mixed at 37°C, layered onto the first layer, and covered with 24x60 mm coverslips. After waiting for them to solidify at +4°C, the coverslips were carefully removed to prepare the slides. Sperm samples were embedded in agarose gel on the slides, and then, the slides were incubated for approximately 1 hour at +4°C in a newly prepared cold lysis solution containing high salt and detergent (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, 1% Triton X-100, pH 10). Afterward, 40 mM DTT was added, and incubation was continued for one more hour at +4°C. Finally, 100 µg/ml proteinase K was added to the slides, and incubated overnight at 37°C. To separate the DNA strands, slides were incubated in fresh and pre-cooled electrophoresis buffer for 20 minutes. Following the incubation, the DNA underwent electrophoresis at 300 mA and 20 volts in an electrophoresis buffer (comprising 300 mM NaOH and 1 mM EDTA, pH 12.5) on the slides. To eliminate the alkaline electrophoresis solution from the slides, a thorough washing was carried out three times using tris buffer (containing 40 mM Tris HCl, pH 7.4) after the electrophoresis process. Following neutralization with ethidium bromide (5 µg/ml), DNA strands were stained, and results were evaluated within 4 hours. The slides were covered with coverslips, and 100 DNA strands were assessed

under a fluorescence microscope (Olympus CX-31) at 400x magnification. DNA damage was determined using the comet assay method and evaluations were performed with TriTek CometScore software version 1.5 (TriTek Corp., Sumerduck, VA, USA). In our evaluation of DNA damage, tail length, tail moment, and tail DNA percentage were analyzed. All procedures were performed in a dark room to protect against additional chromatin degeneration (Avdatek et al., 2023).

Assessment of Oxidative Stress Parameters

The assessment of malondialdehyde (MDA) levels followed the procedure outlined by Draper and Hadley (1990). The quantification of glutathione (GSH) levels was conducted via spectrophotometry at 412 nm, and the GSH levels were reported in mg/mL using the method by Sedlak and Lindsay, (1968). The total antioxidant status (TAS) and total oxidant status (TOS) were determined using an automated novel colorimetric measurement method.

Statistical Analysis

Before conducting significance tests, all variables underwent a normality assessment through the Shapiro-Wilks test, and the homogeneity of variances was evaluated using Levene's test. Potential differnces were statistically examined for variables demonstrating a normal distribution, employing a one-way variance analysis (ANOVA). In cases where significant distinctions emerged among groups for certain variables, post-hoc analyses were carried out using the Duncan test. The statistical analysis was executed utilizing SPSS 22.0 software, and statistical significance was established at $P < 0.05$ and $P < 0.001$ levels.

RESULTS

Table 1 presents data regarding sperm motility and rates of abnormal spermatozoa. Concerning motility, a statistically significant improvement ($p < 0.05$) was observed in the group administered PA10 compared to the C group. Furthermore, there was a statistically significant reduction in head, tail, and overall abnormal spermatozoa rates in the group receiving PA10 compared to the C group. A statistically significant decrease in midpiece abnormalities was evident in the groups receiving PA10 and PA25 ($p < 0.05$). The semen samples tested were 18 (n=18).

Table 2 presents the results of the HE test, revealing notable findings. The group administered PA10 exhibited a significant increase in H+/E- values compared to the C group. Significant reductions in

Table 1. Motility and abnormal spermatozoa ratio after freezing-thawing (± SEM, n:18).

Groups	Motility (%)	Head (%)	Mid-Piece (%)	Tail (%)	Total (%)
Control	65.41±1.64[b]	2.16±0.21[a]	2.50±0.26[b]	8.67±0.40[a]	13.33±0.42[ab]
10 µg/ml	74.17±1.54[a]	1.00±0.13[b]	0.92±0.08[d]	6.33±0.38[b]	8.25±0.49[c]
25 µg/ml	45.42±1.19[c]	2.00±0.45[a]	1.92±0.38[c]	7.75±0.28[a]	11.66±0.40[b]
50 µg/ml	40.00±1.71[d]	2.33±0.26[a]	2.83±0.10[ab]	8.50±0.77[a]	13.66±0.31[ab]
100 µg/ml	31.66±1.24[e]	2.08±0.73[a]	3.08±0.24[a]	8.75±0.88[a]	15.50±1.53[a]
p	*	*	*	*	*

a-e: The differences between values with different letters in each column are statistically significant. p<0,05

Table 2. HOST/E-test values after freezing-thawing (± SEM, n:18).

Groups	H+/E- (%)	H-/E- (%)	H+/E+ (%)	H-/E+ (%)
Control	39.83±2.26[b]	29.33±2.03[a]	17.00±1.55[c]	13.83±1.87[c]
10 µg/ml	46.00±1.15[a]	33.17±1.22[a]	10.17±1.45[d]	10.67±0.80[c]
25 µg/ml	26.00±2.17[c]	21.17±1.83[b]	28.67±3.42[b]	24.17±2.07[b]
50 µg/ml	21.16±0.60[d]	16.17±0.31[c]	36.33±2.17[a]	26.67±1.72[b]
100 µg/ml	14.66±2.06[e]	12.33±0.49[c]	38.67±0.67[a]	34.33±2.48[a]
P	*	*	*	*

a-e: The differences between values with different letters in each column are statistically significant. p<0,05 H+/E- (swollen tail, no dye in the head), H-/E- (unswollen tail, no dye in the head), H+/E+ (swollen tail, dye in the head), and H-/E+ (unswollen tail, dye in the head)

H-/E- values were observed in the groups receiving PA25, PA50, and PA100 compared to the C group. As for H+/E+ values, a noteworthy decrease was detected in the PA10 group, whereas other groups showed an increase relative to the C group. Lastly, the H-/E+ values demonstrated a significant increase in the groups receiving PA25, PA50, and PA100 compared to the C group ($p < 0.05$). The semen samples tested were 18 (n=18).

As depicted in Table 3, the DNA damage results reveal noteworthy insights. Concerning DNA damage, we observed significant reductions in tail length for the groups administered PA10 and PA25, in tail DNA for those receiving PA10, PA25, and PA50, and in tail moment for all groups supplemented with antioxidants ($p < 0.001$). The semen samples tested were 18 (n=18).

Table 4 provides data on oxidative stress parameters. MDA, GSH, TAS, and OSI did not exhibit statistically significant differences across the groups. However, TOS significantly increased in the groups that received PA10, PA25, and PA50 compared to the C group ($p < 0.05$). The semen samples tested were 18 (n=18).

DISCUSSION

The lower phospholipid-cholesterol ratio in ram spermatozoa makes them more vulnerable to oxidative damage, which affects post-thaw motility (Avdatek and Gündoğan, 2018). Spermatozoa are particularly susceptible to oxidative stress due to their limited cytoplasmic space and high levels of polyunsaturated fatty acids, which increase their vulnerability to ROS and lipid peroxidation (İnanç et al., 2019). This oxidative damage impairs sperm motility, DNA integrity, and viability, and is linked to lipid peroxidation, which negatively impacts sperm function (Aitken et al., 2012; Taşdemir et al., 2020). The levels of MDA, a byproduct of lipid peroxidation, serve as an indirect measure of oxidative stress and free radical damage to cells (Fraczek et al., 2001). Antioxidants incorporated into sperm extenders can mitigate lipid peroxidation and oxidative stress, improving overall sperm quality (Salami et al., 2016). Our study revealed a noteworthy enhancement in

Table 3. DNA Damage findings after freezing-thawing ($\overline{x}$± SEM, n:18)

Gruplar	Tail Lenght (µm/s)	Tail DNA (%)	Tail Moment (µm/s)
Control	38.31±1.06^{a}	40.80±0.67^{a}	28.06±0.83^{a}
10 µg/ml	31.24±1.28^{c}	35.58±0.60^{b}	22.32±0.78^{b}
25 µg/ml	32.88±1.13^{bc}	36.11±0.63^{b}	23.12±1.14^{b}
50 µg/ml	36.08±0.88^{ab}	36.63±0.75^{b}	23.27±0.87^{b}
100 µg/ml	36.03±1.40^{ab}	39.82±0.58^{a}	23.61±1.08^{b}
P	**	**	**

a-c: The differences between values with different letters in each column are statistically significant. p<0,001 **

Table 4. Oxidative stress parameters after freezing-thawing ($\overline{x}$± SEM, n:18).

Groups	MDA nmol/ml	GSH mg/ml	TAS (mmolTrolox Equiv./L)	TOS (µmol hidrojenperoksit-Equiv./L)	OSI
Control	4.10±0.18	0.38±0.002	6.77±1.21	13.19±0.38^{b}	20.11±1.30
10 µg/ml	4.98±0.32	0.38±0.003	7.09±0.78	15.60±1.05^{a}	22.31±2.10
25 µg/ml	4.56±0.53	0.39±0.008	7.04±0.35	17.03±0.29^{a}	24.24±0.87
50 µg/ml	4.29±1.52	0.39±0.001	6.75±0.40	13.47±0.78^{b}	19.92±1.75
100 µg/ml	4.83±0.97	0.39±0.000	7.37±0.12	15.74±0.72^{a}	21.46±0.80
p				*	

a-b: The differences between values with different letters in each column are statistically significant. p<0,05 *

motility within the group supplemented with PA10 in the post-thaw solution, as compared to the C and the other experimental groups ($p<0.05$). Additionally, a statistically significant decrease ($p<0.05$) was observed in both the head and tail. And overall abnormal spermaotozoa percentages were observed in the PA10 group, alongside a reduction in midpiece anomalies compared to the C group. PA, such as those obtained from grapes, are rich in antioxidants, effectively scavenging harmful free radicals. These compounds are beneficial in reducing damage to lipids embedded in cell membranes and LDL-derived lipid peroxidation (Evans and Maxwell, 1987). The incorporation of PA into the sperm extender has demonstrated a dose-dependent improvement in sperm motility and membrane integrity, aligning with findings from prior research utilizing various antioxidants (Bucak and Tekin, 2006; Tuncer *et al.,* 2010; Avdatek and Gündoğan, 2018; Güngör *et al.,* 2019). The stability of the sperm plasma membrane is essential not only for sperm metabolism but also for acrosome reaction and capacitation. We observed a noteworthy rise in the H+/E- ratio and a reduction in the H+/E+ ratio within the PA10 group - compared to C ($p < 0.05$). The H/E test, which concurrently assesses the ratios of live and dead sperm and membrane integrity, is a crucial fertility parameter (Avdatek *et al.,* 2020). Our investigation revealed that the supplementation of PA to the sperm extender substantially conserved membrane integrity in a dose-dependent manner. Concerning DNA damage, we observed noteworthy reductions in tail moment, tail DNA, and tail length values in the groups supplemented with PA10, PA25 and PA50 compared to the C ($p < 0.001$). Regarding TOS in spermatozoa, there was a significant increase in the groups supplemented with PA10, PA25 and PA100 compared to the C ($p<0.05$). Our findings from the post-thaw results indicated that the group containing PA10 provided the most effective protection for sperm motility, the proportion of abnormal and live-dead spermatozoa, membrane integrity, and DNA damage.

Our findings align with previous studies that have explored the effects of proanthocyanidin (PA) supplementation on sperm quality, particularly in ram semen. For instance, Avdatek et al. (2020) demonstrated that PA supplementation at 10 µg/ml in Merino ram sperm extenders significantly improved sperm motility and membrane integrity, which mirrors the positive effects observed in our study, where PA10 supplementation provided the most effective protection against oxidative damage and enhanced sperm motility post-thaw. Similarly, in a study by Ven et al. (2019), PA supplementation improved motility and membrane integrity in goat sperm extenders, which further supports our observation that PA enhances spermatozoa quality in different species.

Although our study focused on ram semen, the consistency of PA's beneficial effects across species is notable. The ability of PA to enhance sperm motility, reduce abnormal spermatozoa percentages, and protect membrane integrity may be due to its antioxidant properties, which help mitigate the oxidative stress caused by cryopreservation. This finding is consistent with the results of Zhao et al. (2017), who reported that grape pomace, a rich source of polyphenols, improved sperm quality in rams exposed to oxidative stress. Moreover, the significant reduction in midpiece anomalies observed in our study mirrors the results reported by Li et al. (2018), who found that PA protected sperm membranes from oxidative damage in pig sperm.

The results from the comet assay, presented in Table 3, reveal significant reductions in tail length, tail DNA, and tail moment in the 10 µg/ml PA-treated group when compared to the control group, indicating a marked reduction in DNA damage after the freezing-thawing process. Specifically, the 10 µg/ml PA group exhibited the lowest values for tail length (31.24±1.28 µm), tail DNA percentage (35.58±0.60%), and tail moment (22.32±0.78 µm), all of which point to a reduction in DNA fragmentation and oxidative damage. These findings are consistent with those of Avdatek et al. (2023), who suggested that PA supplementation significantly mitigates DNA damage. Furthermore, the 25, 50, and 100 µg/ml PA-treated groups also showed improvements compared to the control; however, they did not achieve the same extent of DNA protection as the 10 µg/ml group, highlighting a dose-dependent protective effect of PA on sperm DNA integrity during cryopreservation. The reduction in tail length, tail DNA percentage, and tail moment directly correlates with decreased oxidative stress, as these parameters reflect the extent of DNA strand breaks and oxidative damage caused by free radicals during sperm freezing and thawing. Our results are further supported by previous studies that attribute PA's protective effects to its antioxidant capacity. For example, Zhao et al. (2014) found that PA supplementation reduced oxidative damage and improved sperm quality in rats exposed to cisplatin-induced stress, suggesting a similar protective mechanism in our study. This

antioxidant action likely contributed to the observed decrease in sperm DNA fragmentation, which has important implications for fertility and assisted reproductive technologies. In summary, our findings provide strong evidence that PA supplementation, particularly at 10 µg/ml, significantly reduces DNA damage in ram sperm following cryopreservation, likely through its antioxidant properties that neutralize free radicals and protect sperm DNA integrity.

In contrast to the extensive studies on rats (Attia et al., 2010; Su et al., 2011; Bai et al. 2014; Zhao et al. 2014; Sönmez and Tascioglu, 2016; He et al., 2017; Long et al., 2017; Bashir et al., 2019) and poultry (Al-Daraji, 2012), which may involve different oxidative stress conditions, our work provides focused evidence on ram spermatozoa, directly linking PA supplementation to improvements in post-thaw sperm quality. The species-specific differences are crucial when interpreting these findings, as rams and bucks are particularly vulnerable to oxidative damage due to the unique composition of their sperm membranes, which contain a higher proportion of polyunsaturated fatty acids (Avdatek and Gündoğan, 2018). This vulnerability likely makes PA supplementation particularly beneficial in preventing oxidative damage during cryopreservation in these species.

Overall, our study supports the idea that PA, particularly at 10 µg/ml, has a dose-dependent protective effect on ram sperm quality by enhancing motility, reducing sperm abnormalities, preserving membrane integrity, and protecting DNA from oxidative damage. This protective effect is consistent with previous studies, which have demonstrated that PA scavenges free radicals such as ROS and hydroxyl radicals (Wood et al., 2002), and can effectively remove superoxide anions and hydroxyl radicals, interrupting free radical chain reactions (Fracassetti et al., 2013). These results align with findings from other studies on rams and bucks, emphasizing the role of PA as a potent antioxidant that could significantly improve sperm preservation techniques in these species.

CONCLUSION

In conclusion, the addition of 10 µg/ml proanthocyanidin (PA) to ram semen diluent provided the most effective protection for semen quality during cryopreservation. This concentration significantly improved sperm motility, reduced abnormal spermatozoa rates, and preserved membrane integrity and DNA, making it the optimal dose for enhancing the quality of stored ram semen.

Authorship contribution statement

Methodology, SBB, F.A; investigation, SBB, F.A; resources, SBB; writing—original draft preparation, SBB, F.A; writing—review and editing, F.A; project administration, F.A All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors affirm that they have no known financial or interpersonal conflicts that would have seemed to impact the research presented in this study.

Data availability

Data will be supplied upon request.

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