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The Effect of Resveratrol and Catalase on Post-Thaw Angora Buck Semen

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ABSTRACT

This research aimed to examine the impact of resveratrol and catalase on the motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity of Ankara buck semen following freeze-thawed process. In this study, semen samples obtained from four mature bucks were divided into four groups: control (C), resveratrol 500 µM/ml (R), catalase 50 IU/ml (CAT), and resveratrol 500 µM/ml + catalase 50 IU/ml (CATR). After dilution with Tris/egg yolk extender, the semen samples were frozen in liquid nitrogen and then thawed for assessment. The CATR group gave the highest values across all evaluated parameters (motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity) compared to the other groups (61 ± 1.0%, 72.6 ± 0.70%, 70.73 ± 0.67%, 60.9 ± 0.79%, respectively) ($p<0.05$). In conclusion, the combination of catalase and resveratrol significantly improved the quality of buck semen after freeze-thawed process, thereby contributing to enhanced reproductive outcomes and genetic preservation.

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Introduction

Goats have a significant historical background among domesticated animals, known for their ability to adapt to challenging terrains. Their agile and curious nature makes them well-suited for various habitats. Goats can thrive on a wide range of plant materials, including grasses, leaves, twigs, and shrubs. As efficient grazers, they often consume vegetation that other animals find unpalatable. Their exceptional resilience and survival skills enable them to live in diverse regions. In addition to providing animal proteins such as meat and milk for human consumption, goats also contribute to the leather and textile industries. Due to their low water and feed requirements, goats can be effectively raised in arid and semi-arid areas, enhancing their resistance to changing global climate conditions. Despite ranking third in global milk production, goat milk is preferred by a larger demographic compared to milk from other animals (Hussein et al., 2023; Raheem et al., 2024).

Semen extenders are source of energy for sperm cells, protect them against cold shock, and create an optimal environment for cryopreservation. However, the cryopreservation of buck semen remains within reproductive techniques. Both Tris and citric acid are effective buffers for preserving buck and bull semen (Mishra et al., 2010; Purdy, 2006). The primary semen extenders used for bucks are formulated with either egg yolk or skimmed milk powder. bucks produce a specific lipase enzyme upon ejaculation that reacts with the lipids in egg yolk and triglycerides in skim milk, generating harmful compounds for spermatozoa (Sias et al., 2005). This phenomenon is unique to buck semen, and the relevant lipase enzyme is identified as egg yolk coagulating enzyme (EYCE) (Ranjan et al., 2022). The decline in sperm motility observed in bucks when milk-based extenders are used might be attributed to SBUIII, a protein from the bulbourethral gland, also known as triacylglycerol

lipase (Pellicer-Rubio et al., 1997). During the cryopreservation process, potential damage to sperm DNA, acrosomal disruptions, membrane lipid peroxidation (Aitken et al., 2020), loss of mitochondrial potential (Shah et al., 2016) and apoptotic changes (Agarwal & Majzoub, 2017) may occur. These issues are linked to excessive production of reactive oxygen species (ROS), leading to diminished sperm viability and motility, reduced fertilization and pregnancy rates, compromised embryo quality, and hindrances in blastocyst development (Simon et al., 2019). To mitigate these damages, it is crucial to establish a balance in combating ROS generation (Khalique et al., 2023). Consequently, there is a growing interest in using compounds like resveratrol and catalase, which are exogenously administered antioxidants aimed at preventing oxidative harm (Al-Mutary, 2021).

Resveratrol, also known as 3,5,4-trihydroxystilbene, is a stilbenoid and a natural polyphenol with antioxidant properties. This compound can reduce the production of reactive oxygen species (ROS) in mitochondria, scavenge superoxide radicals, mitigate apoptosis, and prevent lipid peroxidation. Additionally, it can regulate the expression of antioxidant cofactors and enzymes (Li et al., 2018; Pervaiz & Holme, 2009). Adding resveratrol to extenders has been shown to reduce lipid peroxidation, protect sperm cells, enhance phosphorylation of 5′AMP-activated protein kinase, decrease ROS generation, and improve the antioxidative defense mechanism of thawed semen (Zhu et al., 2019). Consequently, resveratrol is used to improve semen quality in various animal species, such as bulls (Bucak et al., 2015; Li et al., 2018; Ugur et al., 2019), buffaloes (Longobardi et al., 2017), bucks (Lv et al., 2019), and boars (Zhu et al., 2019). It has been observed to positively impact semen motility and mitochondrial function in different species, including humans (Shabani Nashtaei et al., 2017), rats (Ourique et al., 2013), and roosters (Najafi et al., 2019) protect DNA integrity, and enhance in vitro sperm penetration and fertilization rates.

Catalase, a widely occurring enzyme in nature, primarily facilitates the decomposition of hydrogen peroxide (H_2O_2) into oxygen and water. Beyond its catalytic role in breaking down H_2O_2, catalase can also mediate the oxidation of electron donors like ethanol or phenols at low concentrations. Since hydrogen peroxide levels differ among tissues, catalase's function can vary across different tissues and subcellular compartments (Aitken, 1995; Percy, 1984). By degrading H_2O_2, catalase prevents chain reactions leading to lipid peroxidation and the generation of other reactive radicals (Fernández‐Santos et al., 2009). Thus, catalase is recognized as one of the enzymatic defense systems naturally present in semen, protecting sperm cells from oxidative damage (John Aitken et al., 1989). Catalase's effect on sperm is crucial for reducing their vulnerability to lipid peroxidation during freezing, minimizing the adverse effects of cold shock, halting sperm aging, prolonging their lifespan, and preserving sperm quality (Asadpour et al., 2011).

The main purpose of this study is to evaluate the effects of resveratrol and catalase on buck semen parameters after freeze-thawed process. The hypothesis is that these antioxidants can protect semen motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity during freezing and thawing.

Materials and Methods

Reagents

All chemical compounds and reagents used in the study, including Tris base (T6066), citric acid (C0706), fructose (F2543), glycerol (G2025), Catalase (C1345), Resveratrol (R5010), and FITC-PNA (L7381), were obtained from Sigma-Aldrich (St. Louis, MO, USA), Biological Industries (1% Penicillin-streptomycin-amphotericin B combination, 03-033-1B), and Thermo Fisher Scientific (Live/Dead™ Viability Kit L7011, JC-1 T3168). The preparation of resveratrol was adapted from Bucak et al. (2015), and the preparation of catalase was adapted from Shafiei et al. (2015).

Collection of Semen

Four adult bucks, aged between 2 and 5 years, were used in the study. The care and feeding of the animals were conducted at the Selçuk University Veterinary Faculty, Prof. Dr. Hümeyra Özgen Research and Application Farm. The experiments were carried out during the breeding season of the bucks. Ejaculates were collected three times a week using an electroejaculator (P-T Electronics, Model 302, Boring, Oregon, USA). The ejaculates were then subjected to mass and motility evaluations. Ejaculates scoring 3 or higher in mass and exceeding 80% motility were pooled and transferred to a 37°C water bath. A Tris-based semen extender (Tris 297.8 mM, citric acid 96.32 mM, fructose 82.66 mM, 20% egg yolk, 5% glycerol; pH: 7, 300 mosm) was used as the main diluent for the semen. The pooled ejaculates were divided into four equal parts.

- Control (C)
- Resveratrol 500 µM/ml (R)
- Catalase 50 IU/ml (CAT)
- Catalase 50 IU/ml + Resveratrol 500 µM/ml (CATR)

The samples were diluted in Tris/egg yolk extender at 37°C to a concentration of approximately 400 million spermatozoa per milliliter. After dilution, the semen samples were loaded into 0.25 ml French straws and allowed to equilibrate at +4°C for 3 hours. Following the equilibration process, the semen samples were frozen in liquid nitrogen vapor (~-100°C) for 15 minutes and then stored in liquid nitrogen at -196°C. Twenty-four hours after the freezing process was completed, the samples were thawed at 37°C for 25 seconds and evaluated for semen quality parameters such as motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity.

Mass Activity Score

According to Evans and Maxwell (1987) a drop of the semen sample was placed on a pre-warmed slide and observed under a phase-contrast microscopy (Olympus BX50F4) with a 4x objective lens equipped with a heating stage to evaluate mass activity. The scoring was performed on a scale from 1 to 5.

Motility Examination

Motility was assessed at 37°C using a phase-contrast microscope with a heating stage at 400x magnification. A 10 µl semen sample was placed between a slide and coverslip, and motility was evaluated by examining at least 7 different microscopic fields. The average motility values obtained from these fields were recorded as the motility percentage (Evans & Maxwell, 1987).

Evaluation of Plasma Membrane Integrity

The Live/Dead™ Viability Kit (L7011 Thermo Fisher, Waltham, MA, USA, SYBR-14/PI) was used to assess the viability of spermatozoa (Garner & Johnson, 1995). Thawed semen samples were diluted with PBS (2 x 10^6 spermatozoa/ml). Then, 30 µl of the sample was taken and mixed with 2.5 µl of propidium iodide (PI) (2 mg PI in 1 ml distilled water) and 2.5 µl of SYBR stock solution (diluted 1:5 with DMSO) and incubated in the dark at 37°C for 15 minutes. The samples were then fixed with 10 µl of Hancock solution. Evaluation was performed using a fluorescence microscope (Leica DM 3000). Spermatozoa showing green staining were considered to have intact plasma membranes, while those showing red staining were interpreted as having compromised plasma membranes (Figure 1).

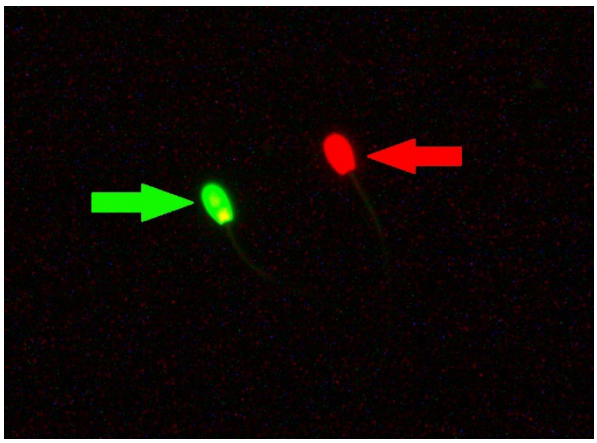

Figure 1. Demonstration of plasma membrane integrity using SYBR-14/PI staining.
The green spermatozoon (green arrow) represents a sperm cell with an intact plasma membrane, while the red spermatozoon (red arrow) represents a sperm cell with a damaged plasma membrane.

Evaluation of Acrosomal Membrane Integrity

To evaluate acrosomal membrane integrity, a modified staining method using Fluorescein isothiocyanate-conjugated Arachis hypogaea/PI (FITC-PNA/PI) as described by Nagy et al. (2003) was applied. In this procedure, 60 µl of sample was mixed with 5 µl of FITC-PNA stock solution (100 µg FITC-PNA in 1 ml PBS) and 2.5 µl of PI solution at 37°C. The mixture was incubated in the dark at 37°C for 15 minutes and then fixed with 10 µl of Hancock solution. Acrosomal membrane integrity was evaluated using a fluorescence phase-contrast microscope (Leica DM 3000). A total of 300 spermatozoa were counted for each group. Spermatozoa showing green fluorescent acrosomes were assessed as having damaged acrosomes, while those without green fluorescence were considered to have intact acrosomes (Figure 2).

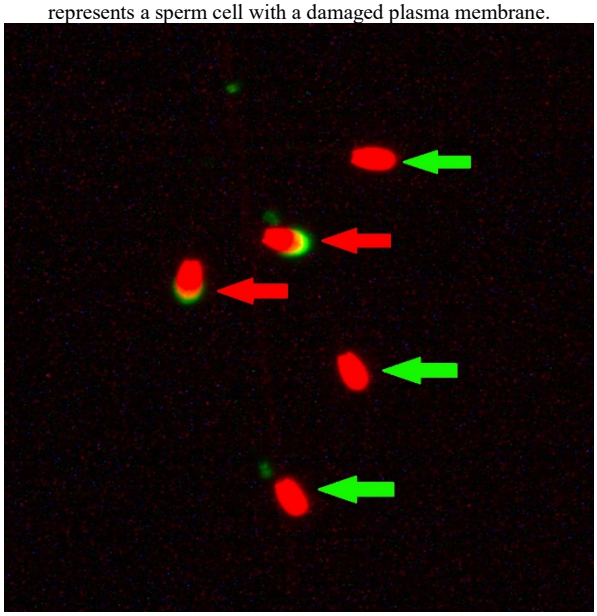

Figure 2. FITC-PNA/PI staining.
Spermatozoa with green-stained acrosomes (red arrow) represent sperm cells with damaged acrosomal membranes, while spermatozoa without green-stained acrosomes (green arrow) represent sperm cells with intact acrosomal membranes.

Evaluation of Mitochondrial Activity

The mitochondrial activity of spermatozoa was evaluated using a modified JC-1/PI staining method adapted from Garner et al. (1997) The JC-1 stock solution (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide, T3168, Invitrogen, 1.53 mM) was prepared in DMSO, filtered, aliquoted (100 µl each), and stored at -20°C. Straws were thawed in a 37°C water bath, diluted 1:3 with PBS, and incubated with 2.5 µl JC-1 and 2.5 µl PI in 300 µl sample. After incubation in the dark at 37°C for 15 minutes, 10 µl Hancock solution was added to stop reaction. The evaluation was performed by placing the semen sample on a microscope slide, covering it with a coverslip, and examining 300 spermatozoa per group under a fluorescence microscope (Leica DM 3000) at 400× magnification. Spermatozoa showing yellow-orange or bright green fluorescence in the midpiece were considered to have mitochondrial activity, while those showing dull green fluorescence were considered to lack mitochondrial activity (Figure 3).

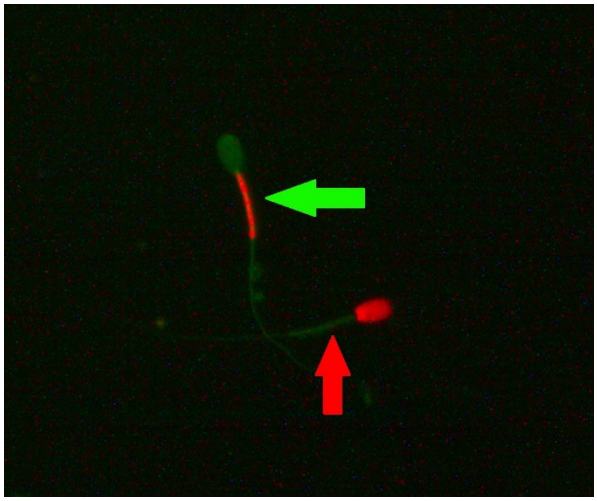

Figure 3. JC-1/PI staining.
A spermatozoon with an orange-stained midpiece (green arrow) represents a sperm cell with mitochondrial activity, while a spermatozoon with a dull green-stained midpiece (red arrow) represents a sperm cell without mitochondrial activity.

Statistical Analyses

The experiment was repeated five times in total. Statistical analyses were conducted using ANOVA followed by Duncan's post-hoc test to determine significant differences for all variables. These analyses were performed using SPSS/PC software version 25.0 (SPSS Inc., Chicago, IL, USA), with statistical significance defined as $p<0.05$.

Results and Discussion

The global increase in goat numbers (FAO, 2023), necessitates preserving genetic diversity and enhancing reproductive efficiency. Cryopreserving buck semen offers significant genetic benefits. Adding antioxidants to cryopreservation solutions can reduce free radical damage and improve semen quality (Bucak et al., 2019; Bucak et al., 2024; Bucak et al., 2020; Karaşör et al., 2022). Until now, studies have evaluated the use of resveratrol or catalase individually. However, there is no study on the effects of their combined use on sperm. This study is the first to investigate the impact of their combined use on sperm. In our study, the CATR group showed significant improvements in these parameters compared to the control group. For the CATR group, motility was 61% ± 1.0, plasma membrane integrity 72.06% ± 0.70, acrosomal membrane integrity 70.73% ± 0.67, and mitochondrial activity 60.9% ± 0.79 ($p<0.05$) (Figure 4).

Resveratrol is a potent antioxidant that reduces oxidative stress in sperm cells by neutralizing reactive oxygen species (ROS) through electron and proton transfer, forming a stable phenoxyl radical (Gülçin, 2010; Leonard et al., 2003). It also enhances endogenous antioxidant enzyme activity, such as SOD, CAT, and GPx, and regulates mitochondrial functions, preserving membrane potential and promoting biogenesis (Said et al., 2012; Zamanian et al., 2023; Zhou et al., 2021). Resveratrol reduces DNA damage, activates repair mechanisms, and prevents apoptosis (de Ligny et al., 2022). Studies show various effective doses: Falchi et al. (2020), found 50 µM/ml improves motility and DNA integrity in buck semen; Lv et al. (2019), found the same dose preserves motility and plasma membrane integrity; Al-Mutary (2019), found 200 µM/ml effective for ram semen; and Kumar et al. (2022), found 0.5 mM best for Hariana bull semen. In our study, group R preserved motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity compared to the control group ($p<0.05$). The group CATR yielded the best results ($p<0.05$). Our study's results are also similar to those of other researchers.

Catalase is a crucial antioxidant enzyme that reduces oxidative stress in sperm cells by converting hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), thus preventing oxidative damage (Chelikani et al., 2004; Said et al., 2012). High levels of H2O2 can damage cell membranes, proteins, and DNA, while catalase protects the structural integrity of sperm membranes by preventing lipid peroxidation, which is vital for sperm motility and fertilization (Aitken & Clarkson, 1987). Catalase also reduces apoptosis caused by reactive oxygen species, improving sperm survival and fertility (Kirkman & Gaetani, 2007; Tremellen, 2008). Studies show varying effective doses. Das et al. (2021), found 200 IU/ml optimal for Black Bengal buck semen; Ranjan et al. (2021), reported 800 IU/ml improved motility, viability, and membrane integrity; Shafiei et al. (2015) found 400 IU/ml beneficial for Bakhtiari buck semen. Gungor et al. (2018), examined the effects of trehalose and catalase on ram sperm after freeze-thawed process. They found that Trehalose 50 mM and Trehalose 50 mM + Catalase 400 µg were the most effective in improving sperm quality. In our study, it was observed that the group CAT provided better protection compared to the control group ($p<0.05$). The group CATR yielded the best results ($p<0.05$). There may be differences among the results obtained by other researchers, but the results are mostly similar. The Tris-based extender and other chemicals used in our study may have different components compared to other studies in the literature, which could explain the differences in the results with different doses of catalase.

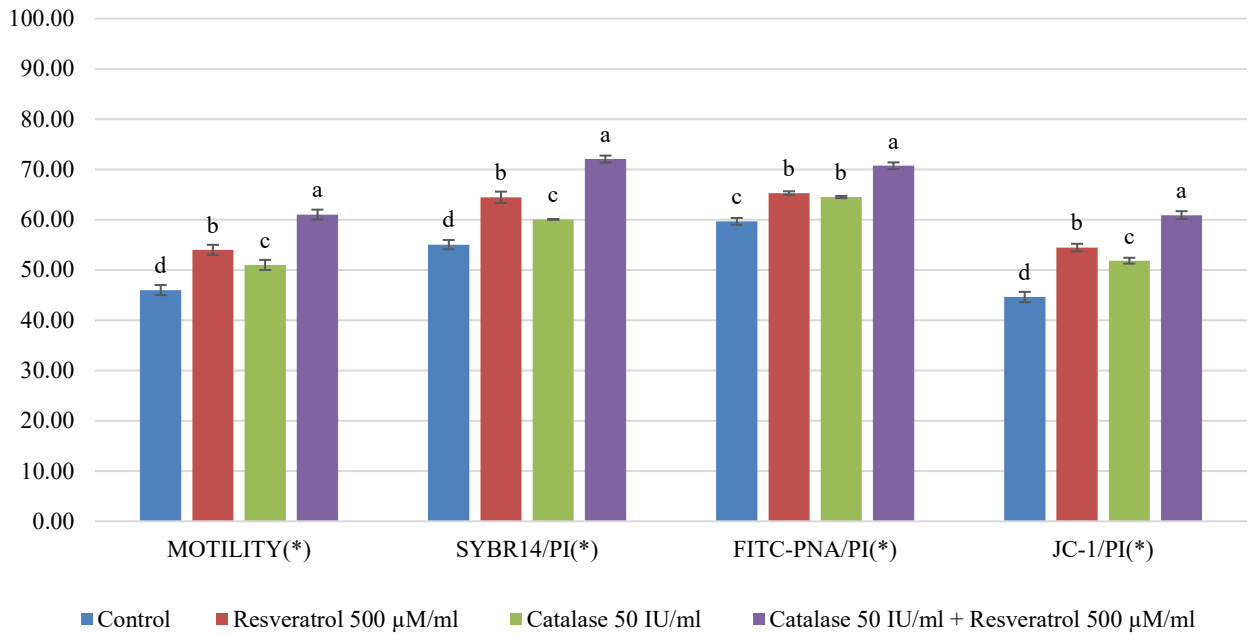

Figure 4. Results of motility, plasma membrane integrity (SYBR-14/PI), acrosomal membrane integrity (FITC-PNA/PI), and mitochondrial activity (JC-1/PI) for the Control, Resveratrol 500 µM/ml, Catalase 50 µM/ml, and Catalase 50 µM/ml + Resveratrol 500 µM/ml groups.

*: Means indicated by different letters (a, b, c, d) within each column are statistically significant ($p < 0.05$).

In our study, it was demonstrated that the combination group CATR had positive effects on sperm parameters after freeze-thawed process of buck semen. The synergy between these antioxidants arises because resveratrol mitigates ROS and oxidative stress, while catalase efficiently handles H_2O_2, a major ROS component. This dual action reduces oxidative stress more effectively than either antioxidant alone, thereby preserving sperm motility, membrane integrity, and overall viability during cryopreservation. These findings are consistent with other studies in the literature, supporting the notion that the antioxidative properties of resveratrol and catalase protect sperm cells against oxidative stress and improve semen quality. Future studies could further expand knowledge in this field by examining the effects of different antioxidant combinations and concentrations on sperm quality in more detail.

Conclusion

This study investigated the effects of resveratrol and catalase on sperm parameters after the freeze-thaw process of buck semen. The group CATR significantly improved sperm motility, plasma membrane integrity, acrosomal membrane integrity, and mitochondrial activity compared to the control. These findings align with existing literature, highlighting the antioxidative properties of resveratrol and catalase in protecting sperm cells and enhancing semen quality. The study contributes to goat breeding and reproductive biotechnology, suggesting that resveratrol and catalase can maximize reproductive success by providing greater protection to buck semen during cryopreservation. Future research should explore different antioxidant combinations and concentrations, their effects on various animal species, and their integration with different cryopreservation protocols.

Declarations

Ethical Statement

This study was approved by (Approval No: 2024/086).

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