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RESEARCH ARTICLE

Commercial Lavender (*Lavandula Angustifolia*) Oil's Effects on Buck Spermatozoa during Cryopreservation

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ABSTRACT

The objective of this study was to assess the impact of lavender oil preparation on the spermatological data, oxidant-antioxidant parameters, and antimicrobial properties of frozen buck semen due to its antioxidant and antibacterial characteristics. To conduct a comprehensive analysis of these impacts, the study established nine experimental groups. Three control groups designed as; a negative control group, a group treated with 1000 IU/mL penicillin and 1000 µg/mL streptomycin (C1), and a group treated with 500 IU/mL penicillin and 500 µg/mL streptomycin (C2). Additionally, groups were created by adding lavender oil to the base diluent. Commercial lavender oil was diluted at a ratio of 10^{-4}. We then formed four groups, each receiving different amounts of this diluted oil: 10 µL/mL, 15 µL/mL, 20 µL/mL, and 25 µL/mL. Simultaneously, two distinct groups were formed by using dosages of 20 µL/mL and 25 µL/mL by adding to the C2 group. The study revealed that lavender oil had protective effects on motility, plasma membrane integrity, and acrosome integrity. Furthermore, lavender oil's antioxidant properties positively affect frozen-thawed semen, and its antibacterial impact was shown when used in conjunction with the C2 group.

Keywords: Antioxidant, Buck semen, Cryopreservation, *Lavandula angustifolia,* Lavender essential oil

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Ticari Lavanta (*Lavandula Angustifolia*) Yağının Kriyoprezervasyon sırasında Teke Spermatozoası Üzerindeki Etkileri

ÖZ

Bu çalışmada, lavanta yağı preparatının spermatolojik veriler, oksidan-antioksidan parametreler ve antioksidan ve antibakteriyel özellikleri nedeniyle dondurulmuş teke spermasının antimikrobiyal özellikleri üzerindeki etkisinin değerlendirmesi amaçlanmıştır. Bu etkilerin kapsamlı bir analizini yapmak için, çalışmada dokuz deney grubu oluşturulmuştur. Üç kontrol grubu; Negatif kontrol grubu, 1000 IU/mL penisilin ve 1000 ug/ml streptomisin (C1) içeren bir grup ve 500 IU/mL penisilin ve 500 ug/mL streptomisin (C2) içeren bir grup oluşturulmuştur. Ek olarak, baz sulandırıcı üzerine lavanta yağı ilave edilen gruplar oluşturulmuştur. Ticari lavanta yağı 10^{-4} oranında seyreltildi. Daha sonra her biri farklı lavanta yağı dozu içeren dört grup oluşturuldu: 10 uL/mL, 15 uL/ml, 20 uL/ml ve 25 uL/mL. Eşzamanlı olarak, C2 grubuna 20 uL/ml ve 25 uL/ml'lik dozlarda lavanta yağı ilave edilerek iki ayrı grup oluşturuldu. Çalışma, lavanta yağının motilite, plazma membran bütünlüğü ve akrozom bütünlüğü üzerinde koruyucu etkileri olduğunu ortaya koydu. Ayrıca, lavanta yağının antioksidan özellikleri dondurulmuş-çözdürülmüş teke sperması üzerine olumlu etkileri olduğu ve C2 grubu ile birlikte kullanıldığında gösterilmiştir antibakteriyel etkisinin olduğu görülmüştür.

Anahtar Kelimeler: Antioksidan, Kriyoprezervasyon, *Lavandula angustifolia*, Lavanta esansiyel yağı, Teke sperması

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INTRODUCTION

Researchers are interested in plant-derived essential oils from aromatic plants because of their antibacterial and antioxidant capabilities. These oils are being studied to assure food safety and protect cellular functions (Danh et al. 2013; Touazi et al. 2018; Valdivieso-Ugarte et al. 2019; Jadczak et al. 2020; Troisio et al. 2024). Lavender oil has various effects including stress and anxiety reduction, promotion of wound healing, treatment of sleep disorders, and use in aromatherapy massage (Cavanagh and Wilkinson 2005; Fismer and Pilkington 2012; Donelli et al. 2019; Samuelson et al. 2020). Furthermore, lavender oil possesses antibacterial, antifungal, and antioxidant properties (Danh et al. 2013).

There are multiple potential sources of contamination while collecting semen using different procedures. Environmental factors and microorganisms introduced into semen may cause adverse impacts on sperm motility, mitochondrial membrane potential, and viability. The impairment of sperm functions and increased microbial load both have the capacity to result in unfavorable outcomes in the female genital tract and increase the risk of reduced fertility (Anel-Lopez et al. 2021). Hence, antibiotics like penicillin, streptomycin, and gentamicin are employed in semen extenders to eliminate the deleterious bacterial action. Still, the presence of reactive oxygen species during the preservation of semen, whether for a short or long period, has negative effects on spermatological parameters. Therefore, semen extenders are supplemented with antioxidants that reduce the harmful effects of reactive oxygen species on spermatozoa (Madeira et al. 2014; Touazi et al. 2018; Ghanem et al. 2023; Troisio et al. 2024).

The objective of this study was to assess the impact of lavender oil, due to its antimicrobial and antioxidant properties, on the microbial load and sperm data following semen freezing. Additionally, the study aimed to investigate the effectiveness of lower doses of antibiotics in semen extenders when combined with lavender oil.

MATERIALS and METHODS

Three Gurcu bucks, aged between 2 and 5 years, were kept and used at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm, which is located at the Faculty of Veterinary Medicine, Kafkas University in Kars, Türkiye. The collection of sperm samples was conducted by veterinarians in accordance with applicable regulations and guidelines governing animal husbandry and welfare.

Experimental Design

The chemicals used in the study were acquired from Merck (Merck, Darmstadt, Germany) and Sigma (Sigma, USA), unless otherwise specified. In the study, we used commercial *Lavandula angustifolia* oil preparation (Talya, Antalya, Turkey) from a company holding U.S. Food and Drug Administration, TS EN ISO 22000, TS EN ISO 9001:2015, ISO 22716:2008 GMP certificates (Talya Bitkisel). Lavender oil was first dissolved in DMSO at a ratio of 1:5 (Lavender oil volume: Total volume) (Do et al. 2021) and then diluted with base semen extender to make the total dilution ratio 1:10,000. Lavender oil diluted to 10^{-4} was used to make all dosages in lavender oil groups. Base semen extender consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA, 4 g/L, 20% egg yolk (v/v) and 6% glycerol (v/v) in distilled water. The study consisted of a total of nine groups: a negative control group (NC) without antibiotics, a group with antibiotics (C1) containing 1000 IU/mL penicillin G potassium (İbrahim Etem, İstanbul, Turkey) and 1000 μg/mL streptomycin sulfate (Menarini, İstanbul, Turkey) in the base extender, an antibiotic group (C2) containing 500 IU/mL penicillin and 500 μg/mL streptomycin, a group in which 20 μL/mL of diluted lavender oil was added to C2 (C20), a group in which 25 μL/mL of diluted lavender oil was added to C2 (C25), a group containing 10 μL/mL of diluted lavender oil (L10), a group containing 15 μL/mL of diluted lavender oil (L15), a group containing 20 μL/mL of diluted lavender oil (L20), and a group containing 25 of diluted μL/mL lavender oil (L25).

The semen collection technique was performed four times using an electro-ejaculator, at a frequency of every other day during non-breeding season. Following the collection, the ejaculates were transported to a water bath maintained at a temperature of 37°C. The evaluation of rapid wave motion and motility was conducted using a phase-contrast microscope (Nikon Eclipse-E400, Tokyo, Japan) equipped with a heated slide set at a temperature of 37°C. Samples exhibiting motility more than 70% and a sperm concentration exceeding $1.5x10^9$ spermatozoa per milliliter were selected for cryopreservation.

Pooled semen was diluted to a concentration of 25 million sperm per milliliter using the appropriate extender. The groups were thereafter chilled to a temperature of 5°C within a time frame of one hour. After being cooled, the sperm samples were allowed to reach equilibrium for a duration of two hours at a temperature of 5°C. The cryopreservation, thawing, and incubation procedures were conducted following the methods described by Yildiz et al. (2015). Three straws were thawed from each group on each study day, and a total of 12 straws were analyzed for each group.

Semen Analysis

The subjective evaluation of sperm motility was conducted using a 400x phase-contrast microscope with a slide warmed to 37°C. The functional integrity of the plasma membrane was assessed using the

hypoosmotic swelling test (HOST), following the approach published by Alcay et al. (2016).

Flow Cytometric Analysis

The analysis was conducted utilizing the Attune NxT Acoustic Focusing Cytometer, manufactured by Invitrogen in the United States. The fluorescence was quantified using a 480 nm excitation wavelength with a 10 nm excitation bandwidth. The emitted light was filtered using a 530/30 nm filter (BL-1) and a 695/40 nm filter (BL-3). The measurements were recorded using Attune NxT software v2.7 (Thermo Fisher). Following the utilization of forward and side scatter light signals to isolate the cell population, the mean fluorescence intensity of the analyzed sperm cells was quantified. The experiment contained a total of 10,000 sperm cells, with a flow rate of 12.5 µl/minute.

The acrosome integrity was assessed using the fluorescein isothiocyanate–conjugated peanut agglutinin (PNA)/propidium iodide (PI) dual-staining approach. The mitochondrial membrane potential was assessed using Rhodamine 123. Flow cytometric analysis was conducted using the methodology published by Gürler et al. (2016).

Biochemical analysis

Thawed semen samples were subjected to spermatological analyses after centrifugation them at 800 g for 10 minutes, resulting in the separation of the supernatant. The malondialdehyde (MDA) level was assessed following the protocol established by Placer et al. (1966), while the reduced glutathione (GSH) level was evaluated using the method described by Sedlak & Lindsay (1968) by using spectrophotometer (Epoch, Biotek, USA). As an MDA standard, 1, 1, 3, 3-Tetramethoxypropane was used, and the results were reported as nmol/mL protein. During the GSH analysis, the samples underwent precipitation using a 10% solution of trichloracetic acid, followed by centrifugation at a speed of 1000 g for 5 minutes. The reaction mixture consisted of 0.5 mL of semen supernatant, 2 mL of tris hydroxymethyl aminomethane buffer (0.4 M; pH 8.9), and 0.1 mL of l 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was maintained at room temperature for a duration of 5 minutes, and subsequently measured at a wavelength of 412 nm using the spectrophotometer. The GSH values were quantified and reported in units of µmol/mL.

Microbiological analysis

For the microbiological analysis, 250 µL semen straws were delivered to the microbiology laboratory under cold chain at 4 ºC. The liquid was transferred into sterile microtubes and then subjected to dilution for the purpose of culture. To achieve this objective, a 10-fold dilution was prepared and then 100 µl of the diluted sample was plated on Plate Count agar up to a 10^5 dilution. The cultures were incubated at 37°C for 18-24h. All analyses were performed in triplicate. Following the incubation period, the number of colonies was determined and the data was recorded. The study results were reported using the logarithm of colony forming units per milliliter (Log_{10} cfu/mL).

Statistical analysis

The power analysis of the study was conducted using the progressive motility results from the control and 0.5 mg/ml zinc oxide nanoparticles groups in the research by Khalique et al. (2023), with a power of 95% and a significance level of 5%. The G*Power® software (Version 3.1.9.7, developed by Franz Faul, University of Kiel, Germany) was used for power analysis.

The statistical analysis was conducted using IBM SPSS version 28. The Shapiro-Wilk test was employed to evaluate the normality of the data. The data were presented as the mean value plus or minus the standard error. The statistical significance of the differences between subdivided groups was assessed using one-way ANOVA followed by Duncan's post hoc test. The Kruskal-Wallis test was employed to analyze data with a distribution that is not normal. Statistical significance was determined for P values below 0.05.

RESULTS

Table 1 presents the rates of motility, plasma membrane integrity, acrosome integrity, acrosome integrity in live cells, and mitochondrial membrane potential. The results for MDA and GSH are provided in Table 2. The findings of the microbiological analysis are shown in Table 3.

Upon thawing, it was found that the L20 and L25 groups exhibited greater preservation of motility compared to the NC group ($p<0.05$), while the C20 and C25 groups shown higher preservation of motility than the C2 group ($p<0.05$). No statistically significant difference was found in the motility values of the L10 and NC groups ($p>0.05$). Also, no statistically significant difference was observed among the motility of the NC, C1, and C2 groups ($p>0.05$). The C20, C25, L20, and L25 groups had higher plasma membrane integrity values compared to the NC, C1, and C2 groups ($p<0.05$).

Significantly greater acrosome integrity values were seen in all groups that used lavender oil ($p<0.05$). Upon analyzing the acrosomal integrity rates in viable cells, it was shown that the acrosome was maintained intact in all groups that included lavender oil ($p<0.05$). The study did not find any significant disparity in the results of mitochondrial membrane potential between the groups ($p>0.05$).

The MDA analysis revealed that the C20, C25, L15, L20, and L25 groups had lower MDA values compared to the NC, C1, and C2 groups ($p<0.05$). A significant difference was also found in MDA values between the L10 group and the NC groups ($p<0.05$). No significant difference in MDA values was found between lavender oil containing groups ($p>0.05$). Glutathione

peroxidase levels were lower in the NC compared to the L10, L15, L20, and L25 groups and lower in C1 and C2 groups compared to the C20, C25 ($p<0.05$). Analysis of the GSH values from the L10, L20, and L25 groups revealed a significant increase in GSH levels in the L20 and L25 groups ($p<0.05$).

The C1 group exhibited the least microbial development following the culture. The C20 and C25 groups had lower levels of growth compared to the C2 group. The NC, L10, L15, L20, and L25 groups did not significantly differ from each other, and more growth was observed as compared to all antibiotic-containing groups.

Table 1. Effect of lavender oil on sperm parameters

Measurements / Groups	Motility (%)	HOST (%)	A (%)	A-P (%)	M (%)
NC	26.25±1.22^{a}	54.17±1.00^{a}	41.13±1.92^{a}	25.91±1.72^{a}	91.35±1.22
C1	27.92±1.61^{ab}	53.58±1.26^{a}	42.18±2.34^{a}	26.05±1.92^{a}	89.79±1.07
C2	29.58±1.99^{ac}	54.50±1.34^{a}	43.26±1.75^{a}	29.86±1.51^{a}	89.60±1.14
C20	35.83±1.49^{d}	60.25±2.38^{bc}	50.52±1.21^{b}	36.26±1.51^{b}	89.91±1.80
C25	37.92±1.89^{d}	63.00±2.71^{c}	52.36±1.83^{b}	37.88±1.97^{b}	91.37±1.34
L10	29.17±1.21^{ac}	53.17±0.84^{a}	53.24±2.27^{b}	36.05±1.31^{b}	91.60±1.06
L15	30.83±0.89^{bce}	57.50±1.31^{ab}	55.63±3.55^{b}	36.25±1.59^{b}	89.14±2.76
L20	32.73±0.66^{cde}	59.70±0.72^{bc}	54.24±3.24^{b}	39.24±3.28^{b}	90.58±1.43
L25	37.08±0.97^{d}	63.50±1.96^{c}	55.83±3.78^{b}	41.49±3.03^{b}	88.99±2.01

$^{a\text{-}e}$: Values with different superscripts in the same column are significantly different ($P < 0.05$). HOST: Plasma Membrane Functional Integrity, A: Total Acrosome Integrity, A-P: Acrosome integrity with Intact Plasma Membrane, M: Total Mitochondrial Membrane Potential

Table 2. Antioxidant effect of lavender oil

Measurements / Groups	MDA (nmol/mL)	GSH (µmol/mL)
NC	120.20±7.79^{a}	12.63±0.68^{a}
C1	114.62±11.00^{ac}	14.11±0.80^{ab}
C2	115.63±7.22^{ac}	14.05±0.37^{ab}
C20	90.58±5.39^{b}	16.58±0.89^{cd}
C25	90.60±6.06^{b}	16.45±0.70^{cd}
L10	97.74±9.89^{bc}	14.92±0.54^{bc}
L15	93.00±10.02^{b}	15.95±0.49^{bcd}
L20	93.88±4.56^{b}	17.28±0.61^{d}
L25	93.15±6.88^{b}	17.06±0.48^{d}

$^{a\text{-}d}$: Values with different superscripts in the same column for each times are significantly different ($P < 0.05$). MDA: Malondialdehyde, GSH: Reduced glutathione

DISCUSSION

In context of the adverse effects associated with synthetic antioxidants, essential fatty acids possessing antioxidant properties have come forward as a significant alternative (Cornwell et al. 1998; Yang et al. 2010). Lavender oil, an essential oil, has demonstrated significant antioxidant activity in free radical scavenging tests. The lipid peroxidation test using linoleic acid, a significant constituent of lavender oil, demonstrated a 58% reduction in peroxidation (Yang et al. 2010). In order to reduce sperm damage during freeze-thaw processes, various enhancements

are implemented in semen extenders (Üstüner et al. 2022; Önder et al. 2023a; Aktar et al. 2024; Önder et al. 2024). Antioxidants are frequently utilized in extenders for this purpose (Avdatek et al. 2018; Bucak et al. 2019; İnanç et al. 2023; Ustuner et al. 2024). In line with these informations, in our study, including lavender oil into the extender enhanced the motility after freezing. While the composition of lavender oil may vary depending on the extraction method (Danh et al. 2013), our research has shown that the commercial lavender preparation helps maintain motility and plasma membrane integrity in NC, L20 and L25 groups and C2, C20 and C25 groups when evaluated individually. However, although the lower doses of lavender oil (L10 and L15) did not show a positive effect on plasma membrane integrity, the first observed positive effects on motility were detected in the L15 group. Semen may contain a diverse range of microorganisms (Moce et al. 2022; Moreira et al. 2022). The presence of these microorganisms have an adverse effect on sperm quality. However, some antibiotics used for this purpose have an adverse effect on the quality of sperm. (Santos and Silva 2020; Moreira et al. 2022). The study revealed notable disparities in bacterial proliferation among the NC, C1, and C2 groups. However, it observed that microbial propagation did not impact motility and plasma membrane integrity. Moce et al. (2022) noted that the quality of sperm can be affected positively or negatively by different types of bacteria. In our study, it was suggested that this difference, contrary to other studies, could also be due to species differences within the microbiota.

Table 3. Antimicrobial effect of lavender oil

Measurements Groups	Microbiological Results (Log_{10} cfu/mL)
NC	4.63±0.06[a]
C1	2.53±0.13[b]
C2	3.83±0.18[c]
C20	3.26±0.14[d]
C25	3.42±0.11[d]
L10	4.63±0.62[a]
L15	4.66±0.04[a]
L20	4.60±0.08[a]
L25	4.50±0.05[a]

[a-d]: Values with different superscripts in the same column for each times are significantly different ($P < 0.05$).

The study found that the addition of lavender oil in adequate quantities to both the group treated with antibiotics and the control group without antibiotics helped maintain the integrity of the acrosome during the semen freezing procedure. Various studies have demonstrated that the use of antioxidants in sperm extenders helps to preserve the integrity of the acrosome (Alcay et al. 2016; Falchi et al. 2020; Toker et al. 2023; Ustuner et al. 2023). On the current basis, the results derived from our investigation exhibit comparable harmony with prior studies on antioxidants. However, despite positive findings compared to the control groups, we found that the mitochondrial membrane potential was similar across all groups. Studies indicate an association with motility and mitochondrial membrane potential (Önder et al. 2023b). According to the results of our study, lavender oil did not have any beneficial effects on mitochondrial membrane potential despite the increased motility. Nevertheless, more thorough research is thought to be needed before a definitive conclusion can be made.

The sperm freezing process leads to the generation of reactive oxygen species, which in turn cause damage to both the structural integrity and functional capabilities of the sperm. The assessment of MDA, resulting from lipid peroxidation, is employed to assess the intensity of oxidative stress (Motlagh et al. 2014). In addition, the assessment of reduced glutathione is employed to evaluate the efficacy of the antioxidant defense system (Kumar et al. 2024). The supposed antioxidant, antibacterial, and other properties of lavender oil are mostly attributed to substances such as linalool, linalyl acetate, 1,8-cineole, cis and trans-ocimene, terpinen-4-ol, and camphor (Kıvrak 2018). In the present study, adding lavender oil to the control groups (NC and C2) resulted in a decrease in MDA levels and an increase in GSH levels. This suggests that lavender oil had a consistent effect on that previous information.

Essential oils have been reported to operate through a multiple mechanism by damaging the cell membrane and suppressing the expression of specific genes (Zych et al., 2024). Several investigations in the literature have assessed the synergistic effects of essential oils, such as

lavender oil, with antimicrobial agents. In these investigations, the interactions between various essential oils and antimicrobial agents are focused on their effects on specific bacterial species (Owen & Laird, 2018). Zych et al. (2020) reported that lavender oil and enrofloxacin had a strong synergistic effect against *Escherichia coli*. Evaluation of the microbiological analyses, in our study, revealed that the inclusion of lavender oil significantly impacted only the groups supplied with low doses of antibiotics. However, when we compared the lavender groups of interest to the negative control group, we found no significant difference. This implies that the dosage used may have been insufficient to detect the antibacterial impact and could have potentially interacted synergistically with the penicillin and streptomycin combination.

CONCLUSION

In conclusion, the study demonstrates that lavender oil is effective in preserving semen through cryopreservation. Additionally, commercially available preparations may serve as a convenient alternative for use.

Author's Contributions: The study was designed, and the manuscript was written by NTO. TG conducted assessments of post-thaw motility and HOST. NTO examined the flow cytometry results. SY and YÖ conducted a semen analysis throughout the process of semen collection. MCK and OS conducted semen collection and cryopreservation and were involved in all semen analysis procedures. SG and MRC performed the microbiological analysis. SA performed MDA and GSH analysis. NTO conducted the statistical analysis.

Ethical Approval: The Scientific Ethical Committee of Kafkas University in Kars, Turkey has granted approval for all matters related to the experimental settings and assessment methodologies (2024-105).

Conflict of Interest: The authors declare that they have no conflict of interest.

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