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Veterinary Sciences *and* Practices

ABOUT

Veterinary Sciences and Practices is a peer-reviewed, open access, online-only journal published by Atatürk University.

Veterinary Sciences and Practices is published triannually in Turkish and English, with issues released in April, August, and December. Beginning on October 17, 2023, Veterinary Sciences and Practices will exclusively consider articles in English. However, Turkish abstracts will still be included in English articles alongside the English abstracts. A language editor will be responsible for translating the English abstracts of accepted papers into Turkish.

Journal History
As of 2022, the journal has changed its title to Veterinary Sciences and Practices.

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Veterinary Sciences and Practices aims to publish studies of the highest scientific level in all fields of veterinary medicine.

Veterinary Sciences and Practices is a comprehensive journal dedicated to the field of Veterinary Medicine and relevant Departments, i.e., Basic Veterinary Sciences (Anatomy, Biochemistry, Pshysiology, Histology, Occupational/Professional Ethics and Deontology), Preclinical Veterinary Sciences (Pharmacology and Toxicology, Microbiology, Parasitology, Pathology, Virology), Clinical Veterinary Sciences (Surgery, Internal Medicine, Animal Obstetrics and Gynecology, Reproduction and Artificial Insemination), Animal Science and Nutritional Sciences (Biostatistics, Genetics, Animal Nutrition and Nutritional Disorders, Animal Enterprises Economy, Animal Science), Animal-Originated Food Hygiene and Technology, with exotic animal science and laboratory animals. The primary focus of the journal is to publish original research that addresses significant clinical inquiries and contributes to the advancement of knowledge and treatment of veterinary conditions. The scope of the journal includes studies on the efficacy of different treatment modalities, innovative diagnostic tools or techniques, and novel approaches to the prevention and management of diverse veterinary diseases and injuries. Veterinary Sciences and Practices aims to foster the dissemination of high-quality research that can enhance the well-being and healthcare of animals, promote animal welfare, and improve the overall practice of veterinary sciences.

Veterinary Sciences and Practices publishes clinical and basic research articles, review articles, systematic reviews articles, and case reports.

The target audience of the journal includes specialists and professionals working and interested in all disciplines of veterinary medicine.

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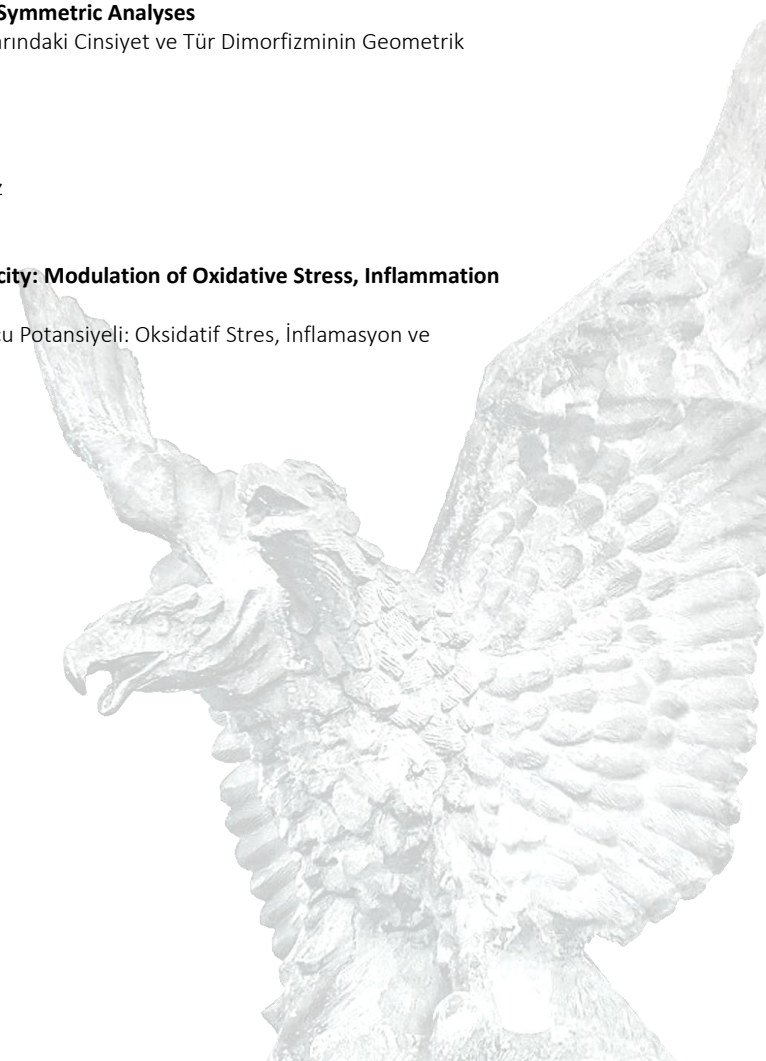

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Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1529099

Protective Effects of Thymol with Hormonal, Anti-inflammatory and Antioxidant Pathways in Lipopolysaccharide-Induced Ovarian Damage in Rats

Sıçanlarda Lipopolisakkarit ile Uyarılan Ovaryum Hasarında Timol'ün Hormonal, Antiinflamatuvar ve Antioksidan Yollarla Koruyucu Etkileri

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ABSTRACT

This study investigated the protective effects of thymol on lipopolysaccharide induced ovarian damage. Female Wistar albino rats were divided into five groups (n=35, group=7): control, vehicle, LPS, thymol, and LPS+thymol. At the end of the study, estradiol 17 beta (E2), anti-Mullerian hormone (AMH), oxidative stress parameters such as malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), catalase, and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) were analyzed in samples taken from animals. Ovary and body weights were also measured. Lipopolysaccharide treatment caused decreases in E2, AMH, GSH, GSH-Px and catalase levels but increased MDA, TNF-α and IL-6 levels. In the Lps+thymol group, thymol administration caused increases in E2, AMH, GSH, GSH-Px and catalase levels and decreased MDA, TNF-α and IL-6 levels. In conclusion, thymol administration positively affected Lps-induced hormonal, oxidative stress and inflammatory changes via antioxidant and anti-inflammatory mechanisms. However, it is thought that the long-term effects of thymol need to be demonstrated, especially by further molecular mechanisms. Within this framework, examining the dose-dependent effects of thymol, the outcomes of its application over varying durations, and investigating the biochemical changes at the cellular level will offer valuable insights for future research and elucidate the potential effects of this compound. These findings reveal the therapeutic potential of thymol; nevertheless, further studies are essential for its clinical application.

Keywords: Inflammation, LPS, oxidant-antioxidant status, ovarian reserve, thymol

ÖZ

Bu çalışmada LPS ile uyarılan ovaryum hasarında timolün koruyucu etkileri araştırıldı. Bu amaçla Wistar albino dişi sıçanlar 5 gruba ayrıldı (n=35, grup=7) : kontrol, taşıyıcı, lps, timol, lps+timol. Çalışma sonunda hayvanlardan alınan numunelerde hormon (E2 ve AMH), oksidatif stres (MDA, GSH, GSH-px ve katalaz), yangısal sitokin (TNF-α, IL-6) analizleri gerçekleştirildi. Ayrıca ovaryum ve canlı ağırlıklarda ölçüldü. Lipopolisakkarit uygulaması, E2 AMH, GSH, GSH-px ve katalaz seviyelerinde azalmalara neden olurken, MDA, TNF-α ve IL-6 düzeylerinde artışa neden oldu. LPS+timol grubunda ise timol uygulaması E2 AMH, GSH, GSH-px ve katalazda artışlara, MDA, TNF-α ve IL-6 seviyelerinde ise azalmalara neden olduğu görüldü. Sonuç olarak timol uygulaması, antioksidan ve anti-inflamatuvar mekanizmalar üzerinden Lps kaynaklı hormonal, oksidatif stres, yangısal değişiklikleri olumlu olarak etkilediği ancak timolün uzun süreli etkilerinin özellikle daha ileri moleküler mekanizmalarla ortaya konmasına ihtiyaç olduğu düşünülmektedir. Bu bağlamda, timolün doz-bağımlı etkilerinin araştırılması, farklı sürelerde uygulanmasının sonuçları ve hücresel düzeydeki biyokimyasal değişimlerin incelenmesi, gelecekteki çalışmalara ışık tutacak ve bu bileşiğin potansiyel etkilerini ortaya koyacaktır. Bu bulgular, timolün terapötik potansiyelini ortaya koymaktadır, ancak klinik uygulamalar için daha fazla araştırma gereklidir.

Anahtar Kelimeler: LPS, oksidan-antioksidan durum, ovarium rezerv, timol, yangı

INTRODUCTION

Microbial environment, metabolites and immune system components coordinate in reproductive system homeostasis.[1] In the postpartum period, the female reproductive tract is highly vulnerable to bacteria, which can easily invade the uterine flora and cause various reproductive pathologies like metritis and endometritis.[2,3] Lipopolysaccharide (LPS), one of the main components of Gram-negative bacteria's outer membrane, can harm reproductive functions such as follicular and embryonic development, ovulation and implantation.[4-7] Also, oxidative stress, inflammation and apoptosis pathways negatively affect ovarian functions like steroidogenesis, folliculogenesis, ovulation, and luteolysis.[8]

Oxidative stress is reported to be involved in the pathogenesis of various female reproductive disorders.[9,10] Lipopolysaccharides cause reactive oxygen species (ROS) accumulation and lipid peroxidation and may damage antioxidant defense systems.[11] It is also known that oxidative stress is associated with inflammation and induces inflammation.[12] The increase in ROS can also stimulate the secretion of tumour necrosis factor-alpha (TNF-α) in oocytes and granulosa cells.[13] In addition, LPS causes immune responses through different molecular pathways.[14] It is known that lipopolysaccharide-induced inflammatory condition causes damage to ovaries and interferes with female reproductive activities.[8] For example, it is stated that inflammatory conditions negatively affect ovarian follicle dynamics.[12]

Thymol, one of the main components of thyme, frequently used in the Mediterranean Diet (MD), is a monoterpene phenolic compound with antioxidant, anti-inflammatory, local anaesthetic, antinociceptive, antiseptic, antibacterial, and antifungal properties.[15] Thymol positively affects the reproductive system in both females and males.[16,17] In a study conducted, thymol caused improvement in sperm parameters in male rats.[16] A study observed high embryo rates in in vitro fertilization in women adhering to the MD.[18] A monkey study showed that the MD caused regular menstrual cycles compared to the Western diet.[19] In addition, the beneficial effects of thymol have been reported in female reproductive disorders such as polycystic ovary syndrome.[20] A study also reported that thymol suppressed the inflammatory response in radiation-induced ovarian damage in rats.[17]

This study aimed to investigate the protective effects of thymol against LPS-induced ovarian damage. For this objective, hormonal, inflammatory, oxidant, and antioxidant status were analyzed, and the protective effects of thymol on female reproductive activities were aimed to be revealed.

MATERIALS AND METHODS

Experimental Design

Female Wistar albino rats (200-250 g) were used in the study (n=35, per 7): control (C), vehicle (V), lipopolysaccharide (LPS), thymol (TML), lipopolysaccharide+thymol (LPS+TML). The experimental model lasted seven days. The TML and LPS+TML groups were given thymol (20 mg/kg/p.o) daily for seven days starting from the first day of the study.[16] Also, the LPS and LPS+TML groups received LPS (100 µg/kg/i.p) four times every second day, starting on the first day of the study, by modifying the model specified by Pal et al.[8] The Vehicle group was given 5% dimethyl sulfoxide (DMSO), used as a thymol solvent, daily throughout the study. (Ethics Committee Decision No: 2023/09-3, Date: 21.12.2023)

At the end of the study, blood was taken from the animals' heart chambers by the intracardiac method under xylazine ketamine anaesthesia. Then, ovarian samples were collected after euthanasia. The obtained blood measured the E_2 and AMH levels. The ovarian tissues measured the levels of TNF-α, IL-6, MDA, GSH, GSH-Px, and catalase. Also, the animals' body and ovarian weights were weighed at the end of the study.

Ovarian and Body Weight Weighing

Right and left ovary samples were thoroughly cleaned, weighed, averaged and expressed in milligrams. Body weights were also weighed and expressed in grams.

Hormone Analyses (E2 and AMH)

Blood samples were centrifuged at 1000 x g for 20 minutes, and sera were collected. E2 levels in the serum samples were measured by electrochemiluminescence immunological method. Serum AMH levels were also measured with a commercial ELISA kit (Finetest, Cat No: ER0260) and expressed as ng/ml.

Ovarian Oxidative Stress Analyses (MDA, GSH, GSH-Px and Catalase)

Both ovaries were brought together and homogenized with 1/10 (weight/volume) Tris-buffered saline (pH 7.4).[17] The homogenate obtained was centrifuged, and the supernatant was collected. All oxidative stress analyses were performed on these supernatant portions.[16] Protein concentrations in the samples were also measured with a commercial BCA assay kit. The Shimadzu UV1700 spectrometer was used for all oxidative stress analyses.

Malondialdehyde levels were measured at 532 nm

according to the method of Placer et al.[22] GSH levels were measured at 412 nm in the spectrometer according to the method of *Sedlak and Lindsay*.[23] MDA and GSH levels are also expressed as nmol/gr protein. GSH-Px was performed according to *Lawrence and Burk*'s method[24], and samples were measured at 340 nm and expressed as IU/gr protein. Catalase was performed according to the method described by Aebi.[25] and expressed as kU/gr protein.

Inflammatory Cytokine Analyses (TNF- α and IL-6)

The ovarian samples (right and left) were homogenized after diluting 1/10 (weight/volume) with phosphate buffered saline (pH 7.4).[17] The homogenate obtained was centrifuged at 5000 g for 5 min at +4°C, and the supernatant was collected. TNF-α (Finetest, Cat No: ER1393) and IL-6 (Finetest, Cat No: ER0042) were measured in this supernatant according to the method specified by ELISA kits. In addition, total protein levels of ovarian tissue were measured by a commercial BCA protein kit. TNF-α and IL-6 levels were expressed as pg/mg protein.

Statistical Analysis

Parametric test assumptions were applied for all variables obtained before proceeding to significance tests. The variables were analyzed using the Shapiro-Wilk test for normality and Levene's test for homogeneity. Then, one-way analysis of variance (ANOVA) was used to control the statistical difference between variables. The Duncan test was used as a post hoc test for the variables in which the difference between the groups was significant. All statistical analyses were performed with a minimum 5% margin of error. IBM SPSS 23 (IBM SPSS Corp., Armonk, NY, USA) was used in all statistical analyses.

RESULTS

Ovarian and Body Weight

The ovarian weights of the C, V, LPS, TML, and LPS+TML groups were measured as 72.514±1.321, 71.690±1.424, 70.043±1.117, 72.062±1.182, and 71.378±1.162, respectively. There was no statistically significant difference between the groups (p=0.690). The mean ovarian weights (Mean±SEM) are shown in Figure 1A.

Body weights of C, V, LPS, TML and LPS+TML groups were 239.714±2.679, 241.429±3.023, 223,571±2.819, 241.714±4.034 and 230.286±2.758 grams, respectively. The lowest body weight averages were in the LPS and LPS+TML groups. Also, the other groups (V and TML) were like the control (P < .001). Body weight averages (Mean±SEM) are shown in Figure 1B.

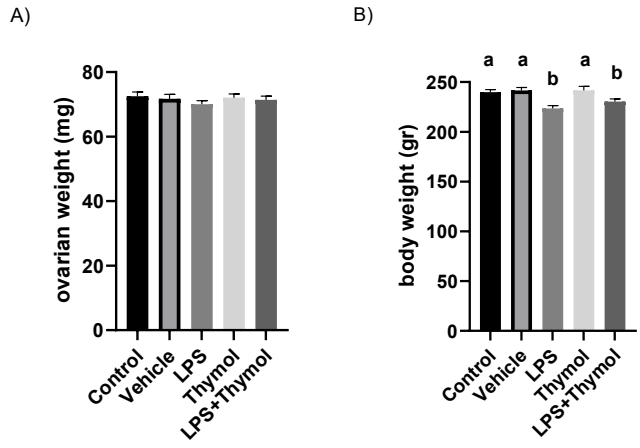

Figure 1. A. Ovary weight averages, **B.** Body weight averages. Letters in the columns indicate statistical differences and similar letters indicate statistical similarity.

Hormone Analyses (E2 and AMH)

E2 hormone levels were 42.351±1.251 in the C group, 42.369±1.535 in the V group, 23.874±1.132 in the LPS group, 43.874±1.901 in the TML group and 33.840±2.327 pg/ml in LPS+TML group. The lowest E2 level among all groups was in the LPS group. In addition, V and TML groups were like the control group, while LPS+TML differed from the control group (P < .001). E2 levels (Mean±SEM) of all groups are shown in Figure 2A.

AMH levels were 1.242±0.041 in the C group, 1.252±0.050 in the V group, 1.058±0.040 in the LPS group, 1.250±0.044 in the TML group, and 1.149±0.047 pg/ml in the LPS+TML group. The LPS group AMH levels were statistically lower than the control group. However, the other groups (V, TML, and LPS+TML) were like the control group (P = .015). The AMH levels (Mean±SEM) of all groups are shown in Figure 2B.

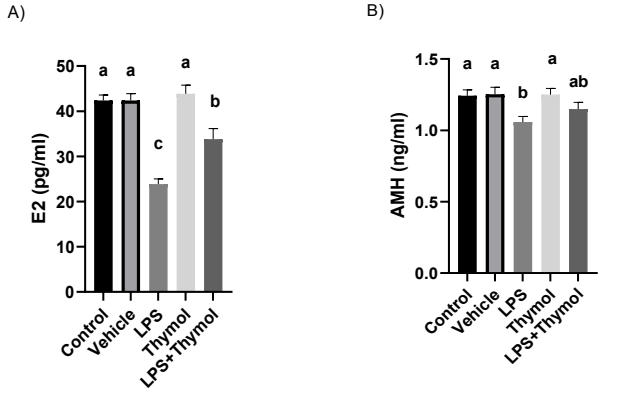

Figure 2. A. Serum E2 levels, **B.** Serum AMH levels. Letters on the columns indicate statistical differences. Similar letters are statistically identical to each other.

Ovarian Oxidative Stress Analyses (MDA, GSH, GSH-Px and Catalase)

Ovarian MDA levels were measured as 2.260±0.045, 2.334±0.100, 3.521±0.057, 2.044±0.073 and 2.836±0.147 nmol/gr protein in all groups, including C, V, LPS, TML, and LPS+TML, respectively. The highest MDA level among all groups was in the LPS group. While V and TML groups were like the control group, the LPS+TML group differed from the control group (P = .001). MDA levels (Mean±SEM) of ovarian tissue are shown in Figure 3A.

GSH levels of the C, V, LPS, TML, and LPS+TML groups were measured as 10.000±0.368, 9.738±0.281, 7.051±0.234, 11.052±0.244, and 8.047±0.153 nmol/gr protein, respectively. The highest and lowest GSH levels were in the TML and LPS groups. While the V group was like the control, the LPS+TML group differed from the control (P < .001). GSH levels (Mean±SEM) of ovarian are shown in Figure 3B.

GSH-Px levels of C, V, LPS, TML and LPS+TML groups were measured as 56.159±2.063, 55.624±1.794, 40.681±1.134, 57.706±1.496 and 47.338±1.538 IU/gr protein, respectively. The lowest GSH-Px levels were in the LPS group. The V and TML groups were like the control, and the LPS+TML group differed from the control (P < .001). GSH-Px levels (Mean±SEM) of ovarian tissue are shown in Figure 3C.

The catalase levels of the C, V, LPS, TML, and LPS+TML groups were 48.336±1.652, 47.069±1.316, 41.359±0.969, 49.748±1.249, and 43.623±1.732 ku/gr protein, respectively. The lowest levels were in the LPS group. While the V and TML groups were like the control, the LPS+TML group was different from the control (P = .001). The catalase levels of ovarian (Mean±SEM) are shown in Figure 3D.

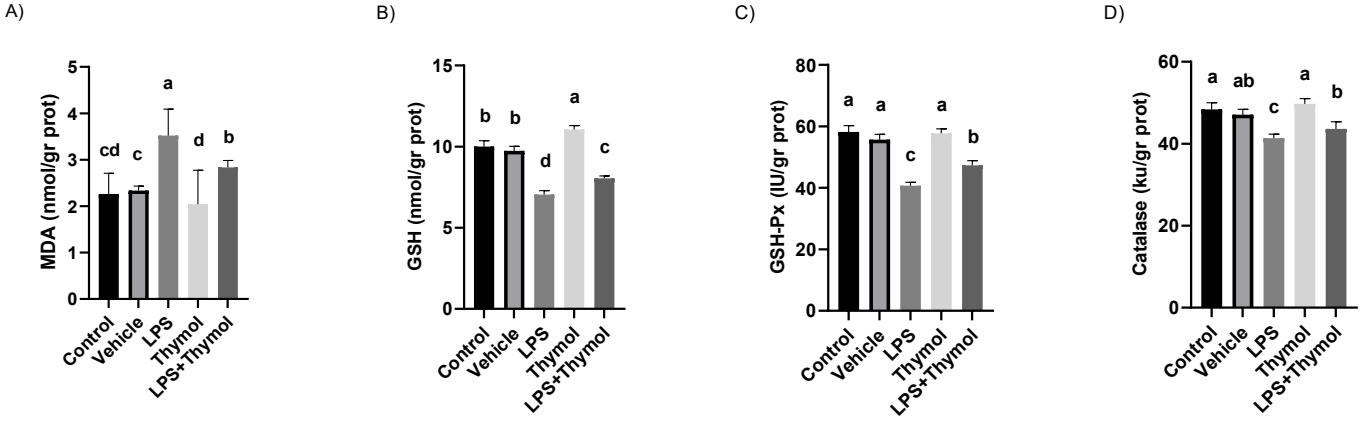

Figure 3. A. Ovary MDA levels, **B.** Ovary GSH levels, **C.** Ovary GSH-Px levels, **D.** Ovary catalase levels. Letters on the columns indicate statistical differences. Similar letters are statistically identical to each other.

Inflammatory Cytokine Analyses (TNF- α and IL-6)

The ovarian TNF-α levels of all groups (C, V, LPS, TML, LPS+TML) were 22.163±0.563, 22.343±0.372, 29.117±1.341, 23.060±0.223 and 25.087±0.733 pg/mg protein, respectively. The highest TNF-α levels were in the LPS group. In addition, while V and TML groups were like the control group, the LPS+TML group differed from the control group (P < .001). TNF-α levels (Mean±SEM) of ovarian tissue are shown in Figure 4A.

The ovarian IL-6 levels of all groups (C, V, LPS, TML, LPS+TML) were measured as 10.001±0.346, 11.104±0.300, 18.813±0.534, 9.187±0.362 and 14.501±0.243 pg/mg protein, respectively. The highest IL-6 levels were in the LPS group. While the TML group was like the control group, V and LPS+TML groups differed from the control group (P < .001). IL-6 levels of ovarian tissue (Mean±SEM) are shown in Figure 4B.

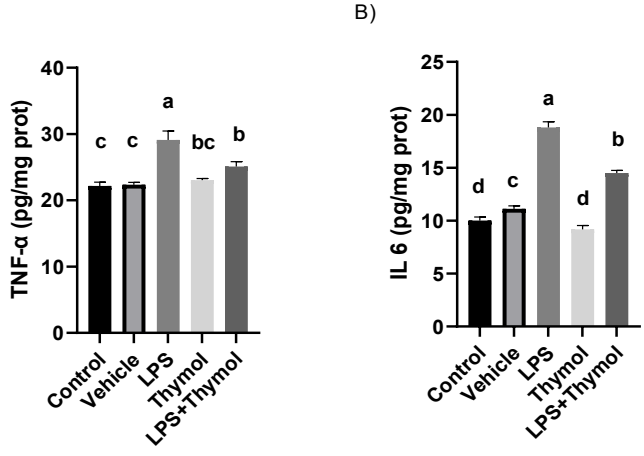

Figure 4. A. Ovary TNF-α levels, **B.** Ovary IL-6 levels. The letters in the columns indicate statistical differences. Similar letters are statistically identical.

DISCUSSION

Although inflammatory processes can lead to follicle depletion, decreased oocyte quality and infertility, controlling this condition positively affects reproductive health.[26-28] Proinflammatory cytokines and reactive oxygen species affect the estrous cycle, steroidogenesis, ovulation, damage oocyte maturation, and embryo development.[29] Previous studies show that LPS cause hormonal imbalances, ovarian failure and infertility in mammals.[30] Similarly, in this study, it is observed that LPS-induced inflammatory condition causes decreases in reproductive hormone levels and disruptions in biochemical parameters in the ovary.

Both low and high doses of LPS prolong the estrous cycle, reduce the number of primordial follicles and change reproductive hormone levels.[31] In a study, it was conveyed that LPS administration caused decreases in serum estrogen and progesterone levels.[8] In another study, it was reported that LPS administration caused decreases in serum estrogen levels.[30] In addition, decreases in LH release occur in inflammation caused by intrauterine LPS.[32] A study stated that LPS administration causes decreases in AMH and E_2 levels in mice.[31] A study even reported that LPS-induced maternal inflammation caused decreases in AMH levels in female offspring mice.[33] In this study, similar to previous studies, LPS administration caused statistically significant decreases in AMH and estrogen levels. Also, it was observed that thymol treatment statistically increased estrogen levels and caused increases in AMH levels.

In a normal estrus, cytokines and chemokines released under physiological conditions are necessary for follicular growth and ovulation.[12,29] Inflammatory mediators are produced during folliculogenesis and participate in ovulation processes.[12] For instance, it is reported that physiological inflammation shaped by the increase in gonadotropin causes weakening and rupture in the follicle wall and contributes to ovulation.[34] C-reactive protein (CRP), another immune system component, is associated with follicular dynamics; women with three follicular waves have higher CRP levels.[35] In addition, bradykinin levels increase approximately 10-fold in ovulation.[34] However, pathological cytokine and chemokine signaling could cause anovulation and infertility by affecting follicular dynamics and impairing oocyte quality.[12,29] Both low and high doses of LPS increase serum and ovarian inflammatory cytokine levels in mice.[31] A study reported decreased follicles with ageing were associated with inflammation in mice.[28] Also, chronic inflammation has harmful effects on follicular dynamics and ovulation.[12] Increasing follicular content inflammatory mediator levels in women receiving infertility treatment causes a decrease in oocytes and a decrease in implantation success.[36] Similar to previous studies, this study found that LPS administration caused statistically significant increases in ovarian TNF-α and IL-6 levels.

In normal physiological conditions, free radicals and antioxidants are in balance, and in oxidative stress situations that occur in the oxidant direction of this balance, oocyte ageing decreases in oocyte quality and quantity, and impairments in embryonic development may occur.[37] Increases in the oxidant level of follicular fluid in women reduce the success of assisted reproductive techniques (ART).[38,39] Also, the granulosa cell antioxidant content of women receiving invitro fertilisation treatment is low.[40] A study observed that LPS administration caused decreases in ovarian superoxide dismutase and catalase levels and increased MDA levels in hamsters.[8] Like other studies, LPS administration caused a statistically significant increase in MDA levels and decreased antioxidant enzyme levels of GSH, GSH-Px and catalase in ovaries.

Antioxidant therapies are among the different protocols for the protection of oocytes.[37] The main principle of treatment strategies involving antioxidants such as antioxidant monomers or melatonin is to reduce oxidative stress and improve ovarian function.[41] Natural antioxidants cause protective effects on ovaries through multiple mechanisms.[42] Thymol, one of the natural antioxidants, can reduce oxidant capacity.[43] For example, a study conducted in rats reported that thymol increased total antioxidant capacity by 115% in radiation-induced oxidative stress.[17] Another study reported that Thymus vulgaris with thymol content showed an antioxidant effect in polycystic ovary syndrome in rats.[44] In line with the previous study, thymol administration in LPS-induced ovarian damage statistically decreased MDA levels, increased antioxidant enzyme levels (GSH-GSH-Px and catalase) and stimulated antioxidant mechanisms.

Identical to antioxidant therapy for oxidant status, controlling inflammatory processes is very important for ovarian activities.[32,36,37] Treatment approaches for controlling follicular fluid cytokine content in women are included in the infertility treatment protocol.[36,45] Upon reviewing the literature, it has been observed that thymol induces anti-inflammatory effects through different molecular mechanisms.[46]Another study stated that the inflammatory condition induced by radiation in the ovary returned to normal levels with thymol in rats.[17] Similar to previous studies, this study observed that using thymol in LPS-induced ovarian damage statistically decreased TNF-α and IL-6 levels in ovarian tissue and thus caused the anti-inflammatory effect.

In conclusion, thymol's use in LPS-induced ovarian damage has protective effects through hormonal, antioxidant, and anti-inflammatory mechanisms. However, further molecular analyses should be performed, and the effects of different doses and durations of thymol on ovarian functions should be investigated.

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Effects of Apilarnil on Semen Parameters During Ram Semen Short-Term Storage

Koç Spermasının Kısa Süreli Saklanması Sırasında Apilarnil'in Sperma Parametreleri Üzerindeki Etkileri

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ABSTRACT

This study aimed to investigate the effects of different concentrations of apilarnil (APL) added to semen diluent on the short-term storage of ram semen. For this purpose, six Akkaraman rams aged 1.5 years were used in the study outside the breeding season. Semen was collected from the rams twice a week with the help of an artificial vagina. Semen was first analyzed and ejaculates with a total motility score of 70% and above were pooled. The pooled ejaculates were divided into 5 equal parts and reconstituted with tris-egg yolk diluent containing 0%, 0.5%, 1%, 1%.5% and 2% APL. Diluted semen samples were stored at 4°C for 72 hours. As a result of the analyses, no statistically significant difference was observed between the groups in terms of total motility at 0 hour, while progressive motility was significantly higher in the 0.5% APL group ($P < .005$). While rapid spermatozoa rate, curvilinear velocity (VCL), and linear velocity (VSL) were high in the control group in all time periods, medium, and slow spermatozoa rates were high in 1% and 1.5% APL groups ($P < .05$). At 0 and 72 hours, the control group had the highest malondialdehyde (MDA) level ($P < .001$). Glutathione (GSH) level and glutathione peroxidase (GSH-Px) and catalase (CAT) activities were significantly lower in the control group at 0 and 72 hours. In conclusion, 0.5-1.5% APL added to semen diluent for short-term storage of ram semen has a positive effect on semen quality and oxidant status.

Keywords: Apilarnil, CASA, cooling, ram, sperm

ÖZ

Bu çalışma, sperma sulandırıcısına eklenen farklı konsantrasyonlardaki apilarnilin (APL) koç spermasının kısa süreli saklanması üzerindeki etkilerini araştırmayı amaçlamıştır. Bu amaçla, çalışmada üreme mevsimi dışında 1,5 yaşında altı Akkaraman koçu kullanıldı. Koçlardan haftada iki kez suni vajina yardımıyla sperma toplandı. Sperma önce analiz edildi ve toplam motilite skoru %70 ve üzerinde olan ejakülatlar pooling yapıldı. Pooling yapılan ejakülatlar 5 eşit parçaya bölündü ve içerisine %0, %0,5, %1, %1,5 ve %2 oranında APL ilave edilmiş tris-yumurta sarısı sulandırıcısı ile sulandırıldı. Seyreltilmiş sperma örnekleri 4°C'de 72 saat boyunca saklandı. Analizler sonucunda, 0. saatte total motilite açısından gruplar arasında istatistiksel olarak anlamlı bir fark gözlenmezken, progresif motilite %0,5 APL grubunda anlamlı olarak daha yüksek bulunmuştur ($P < ,005$). Hızlı spermatozoa oranı, eğrisel hız (VCL) ve doğrusal hız (VSL) tüm zaman dilimlerinde kontrol grubunda yüksekken, orta ve yavaş spermatozoa oranları %1 ve %1,5 APL gruplarında yüksekti ($P < ,05$). 0 ve 72. saatlerde kontrol grubu en yüksek malondialdehit (MDA) seviyesine sahipti ($P < ,001$). Glutatyon (GSH) seviyesi ile glutatyon peroksidaz (GSH-Px) ve katalaz (CAT) aktiviteleri 0 ve 72. saatlerde kontrol grubunda anlamlı derecede düşüktü. Sonuç olarak, koç spermasının kısa süreli saklanmasında sperma sulandırıcısına eklenen %0,5-1,5 APL, sperma kalitesi ve oksidan durumu üzerinde olumlu bir etkiye sahiptir.

Anahtar Kelimeler: Apilarnil, CASA, koç, soğutma, sperm

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INTRODUCTION

Semen is stored at +5°C for insemination because frozen and thawed semen has a low success rate in sheep inseminations.[1] Chilled ram semen exhibits reduced motility and morphological integrity, a shorter time to survive in the female genital canal, and a higher rate of embryonic losses when compared to fresh semen.[2] One of the most important disadvantages of chilled semen is its short fertile life.[3] It is important that the semen is preserved and stored under ideal conditions in order to attain an appropriate fertility rate.[4] Although various diluents have been developed for fresh storage of semen in sheep, semen can maintain its viability for up to 72 hours.[5]

Free radicals are reactive molecules and are spontaneously produced as many by-products during daily metabolic activities.[6] Although free radicals are needed in many biological processes, excessive increase in the amount of free radicals causes oxidative stress.[7] The potential of sperm to fertilize is decreased by oxidative damage.[8] Spermatozoa are highly sensitive to free radicals due to the unsaturated fatty acids present in their cell membranes.[9] Oxidative damage leads to loss of sperm membrane integrity and decreased sperm quality. Antioxidant compounds should therefore be added to the sperm diluent.[10]

The chemical composition of bee products ensures both the nutrition of living organisms and their widespread use in apitherapy.[11,12] Drone brood homogenate (apilarnil (APL)) is obtained from drone larvae between three and eleven days after hatching.[13] APL is produced by homogenizing, filtering, and lyophilizing the drone larvae after they are removed from the comb before pupation.[12] APL has a high protein content and includes essential amino acids.[14] APL contains high antioxidant properties and it has beneficial health effects.[15,16] APL has been reported to be used for the treatment of male infertility as well as systemic problems such as gastrointestinal and respiratory diseases.[17] APL administration has been observed to enhance sperm quality in male rats exposed to BPA-induced toxicity.[18] In the literature review, it is seen that the studies showing the effect of APL in semen diluents are limited. The present study aimed to investigate the effects of APL on the short-term storage of ram semen.

MATERIALS AND METHODS

Ethical approval

The approval certificate (Date: 16.11.2022, Number of Sessions: 2022/18-08) was obtained from Fırat University Animal Experiments Local Ethics Committee.

Study area and design

The animals were housed at Fırat University, Faculty of Veterinary Medicine, Animal Hospital during the study period. They were fed daily with roughage and concentrate feed, and water was provided ad libitum.

Semen was collected from six, 1.5 years old Akkaraman rams during the non-breeding season twice a week through an artificial vagina. Each ejaculate was evaluated by phase-contrast microscope with heating plate (Nikon) for motility, wave motion and concentration. During the wave motion examination, the evaluation is based on the presence or absence of fluctuating motion in the area and, if present, its intensity and velocity. A score between 0-5 is given taking into account the given characteristics. Computer Assisted Semen Analyzer (CASA) was used to determine sperm motility, and concentration. Semen was first analyzed and ejaculates with a total motility score of 70% and above were pooled. The pooled semen was diluted at 35°C in a 1:1 ratio with tris + egg yolk-based semen extender [297.58 mM tris (hydroxymethyl) aminomethane + 96.32 mM citric acid + 82.66 mM fructose + 100.000 IU penicillin + 100 mg streptomycin + 15% egg yolk] and APL dissolved in water. Semen samples divided into 5 groups (control (no additive), 0.5% APL, 1% APL, 1.5% APL, 2% APL). After the semen is diluted, it is placed in a container of water at 35°C and the temperature is reduced to 4°C over a period of 3 hours and the temperature was stabilized. After different doses of APL (Harşena, Amasya, Turkey, Cat. No: 38) were added to the semen, spermatological examinations were performed on semen samples every 24 hours and biochemical examinations were carried out on semen samples 0 and 72. hours.

Oxidative Stress Analysis

The lipid peroxidation (LPO) level was measured according to the concentration of thiobarbituric acid-reactive substances. The amount of malondialdehyde (MDA) was used as the LPO index.[19] Glutathione (GSH) levels were measured using the method described by Sedlak and Lindsay.[20] Glutathione peroxidase (GSH-Px) activities were determined according to the method of Lawrence and Burk.[21] Catalase (CAT) activities were determined by measuring the decomposition of hydrogen peroxide (H_2O_2).[22]Protein concentration was determined by the method used by Lowry et al.[23] Our results were analyzed at the beginning and end of the experiment at 0 and 72 hours.

CASA Analysis

Semen motility analyses were performed using a computer-assisted sperm analyzer (CASA, ISASv1, Proiser, Spain). Semen samples were diluted with tris buffer solution [3.63 g tris, 1.99 g citric acid, 0.50 g glucose in 100 mL distilled water] in an Eppendorf tube at 37°C. 3.5 µL of

the mixture was placed on a Spermtrack20 slide and covered with a coverslip. Total, progressive, rapid, medium and slow motility ratios (%), static sperm ratio (%) and kinematic parameters [VCL: curvilinear velocity (µm/s); VSL: straight line velocity (µm/s); VAP: mean path velocity (µm/s); LIN: linearity index (%); STR: straightness index (%), WOB: wobble index (%); ALH: mean amplitude of lateral head movement (µm); BCF: beat cross frequency (Hz)] were analyzed.[24] All analyses were performed after post equilibration and at 24, 48, 72 hours.

Morphological Analysis

A small amount of semen-tris mixture was placed on a slide, froth was taken and allowed to dry. They were immersed in Diff - Quick staining set solutions for 30 seconds, 20 seconds, and 30 seconds respectively, washed with distilled water and air dried. The smears were then examined under 400x magnification of a phase-contrast microscope. A total of 200 spermatozoa were analyzed in each smear and the results were presented as percentages. Our results were analyzed at the beginning and end of the experiment at 0 and 72 hours.

Statistical Analysis

Before significance tests, the data obtained were analyzed by Shapiro Wilks test for normality and Levene's test for homogeneity of variances. The statistical control of the difference between normally distributed variables was performed by ANOVA, and the control between non-normally distributed variables was performed by Kruskal Wallis test. Tukey's test was used as a post-hoc test for variables in which the difference between groups was significant. Descriptive statistics were calculated for each variable and presented as "Mean ± Standard Error of Mean" (Mean ± SEM). Data were evaluated by two-way mixed design ANOVA (analysis of variance) using the General Linear Model for repeated measures procedure to examine the effect of "group" and "time" for the measurements obtained. Post-hoc testing for significant interactions was performed using simple effects analysis with Bonferroni adjustment. Where interaction terms were not statistically significant, contrasts were used to analyze main effects. All statistical analyses were analyzed with a minimum margin of error of 5%. SPSS 26.0 (IBM SPSS Corp., Armonk, NY, USA) package program was used. $P < .05$ was considered significant.[25]

RESULTS

CASA Evaluation

CASA parameters analysis results are shown in Tables 1 and 2. At the post equilibration, the lowest total motility and progressive motility values were observed in 1.5% and 2% APL groups. Progressive motility values at 24, 48, and 72 hours were highest in 0.5% APL group. At 24, 48, and 72 hours, the highest rate of rapid spermatozoa was seen in the 0.5% APL group and medium spermatozoa was seen in the 0.5% and 1% APL group. VCL and VSL values were highest in the control and 0.5% group at post equilibration and decreased in parallel with the following time periods. LIN, STR, and WOB were higher in the 0.5% APL group compared to the other groups. In general, ALH value was higher in the control group and BCF value was higher in the 0.5% APL group compared to the other groups ($P < .05$).

Table 1. Total motility and progressive motility values (mean±sem) of the experimental groups.

	TM 0	TM 24	TM 48	TM 72	PM 0	PM 24	PM 48	PM 72
Control	80.33±2.53^{Aa}	65.08±2.75^{Ab}	48.18±5.43^{Ab}	33.18±1.43^{Ac}	35.65±3.10^{ABa}	32.9±3.65^{ABa}	14.28±2.36^{Ab}	11.72±1.74^{ABb}
%0.5	83.63±3.07^{Aa}	69.04±4.69^{Ab}	46.13±3.08^{Ac}	35.5±2.44^{Ad}	45.18±2.08^{Ba}	35.72±2.99^{Ab}	16.10±2.81^{Ac}	13.32±0.91^{Ac}
%1	71.72±2.52^{ABa}	64.1±3.14^{Aa}	39.48±3.56^{ABb}	34.43±6.96^{Ab}	30.58±2.73^{ACa}	24.72±3.36^{ABa}	11.08±1.11^{ABb}	11.63±2.37^{ABb}
%1.5	63.48±2.68^{Ba}	53.06±5.41^{Aba}	32.72±0.98^{Bb}	22.97±2.05^{Bc}	24.37±2.41^{CDa}	23.4±2.77^{ABa}	9.52±0.78^{ABb}	6.68±1.10^{BCb}
%2	45.92±5.1^{Ca}	42.00±4.76^{Bb}	25.32±3.63^{Cc}	15.82±0.6^{Cd}	13.37±3.00^{Da}	19.32±2.93^{Ba}	5.05±0.85^{Bb}	3.62±0.55^{Cb}

*A, B, C, D: indicates the difference between groups in the same column ($P < .05$). *a, b, c, d: indicates the difference between times in the same row ($P < .05$).
Abbrevations: TM: total motility, PM: progressive motility

Oxidant/Antioxidant Status Assessment

Mean values of oxidant and antioxidant status at 0 and 72 hours are given in Table 3. MDA levels were significantly higher in the control group compared to the other groups at 0 and 72 hours. GSH levels were significantly higher in all APL groups compared to the control group at 0 and 72 hours. At post equilibration and 72 hours, GSH-Px and CAT activities were highest in 1.5% APL group ($P < .001$).

Abnormal Sperm Rate

Abnormal sperm ratio findings are presented in Table 4. According to this, when 0. hour is analyzed, it is seen that the lowest rate is in 0.5% APL and control group, respectively. The highest value was observed in the 2% APL group ($P < .001$). At 72 hours, it was again observed that the lowest value was in the control and 0.5% APL group, and the highest value was in the 2% APL group ($P < .001$).

Table 2. Sperm kinematic parameters values (mean±sem) according to groups.

Parameters		0. Hour	24. Hour	48. Hour	72. Hour
Rapid (%)					
	Control	66.73±2.35^{Aa}	51.00±3.86^{Ab}	23.27±3.54^{Ac}	15.43±1.57^{Ad}
	0.5 %	68.02±1.48^{Aa}	54.02±5.12^{Ab}	23.65±1.69^{Ac}	17.83±2.10^{Ac}
	1%	48.40±3.25^{Ba}	49.76±4.50^{Aa}	17.68±1.03^{ABb}	12.95±2.06^{ABb}
	1.5 %	36.73±3.21^{Ba}	39.94±5.85^{Aba}	13.42±0.96^{BCb}	8.32±1.75^{BCc}
	2%	22.52±4.17^{Ca}	28.36±4.49^{Ba}	6.72±1.26^{Cb}	2.13±0.32^{Cd}
Medium (%)					
	Control	8.68±0.87^{Aab}	11.18±0.83^{a}	9.48±0.77^{ab}	7.85±0.81^{b}
	0.5 %	8.28±0.57^{Aa}	11.82±0.81^{b}	10.32±1.09^{ab}	6.20±0.59^{c}
	1%	10.88±1.36^{AB}	9.48±0.81	10.12±0.89	8.03±1.71
	1.5 %	13.07±0.78^{Ba}	10.56±0.49^{b}	9.02±0.34^{b}	5.88±0.27^{c}
	2%	11.65±1.27^{ABa}	10.74±0.62^{a}	7.57±0.96^{b}	5.08±0.66^{b}
Slow (%)					
	Control	6.47±0.96^{Aa}	3.92±0.45^{b}	12.10±1.75^{c}	9.90±0.88^{c}
	0.5 %	9.02±0.58^{ABa}	2.74±0.34^{b}	12.15±1.20^{c}	11.28±1.42^{c}
	1%	12.55±1.46^{Ba}	2.88±0.31^{b}	13.40±1.52^{a}	7.7±1.93^{c}
	1.5 %	12.32±0.82^{Ba}	2.54±0.24^{b}	10.25±1.08^{a}	8.73±1.21^{a}
	2%	11.73±1.00^{Ba}	2.92±0.39^{b}	9.33±0.99^{a}	8.57±0.54^{a}
VCL (µm/s)					
	Control	129.80±5.24^{Aa}	102.68±5.60^{Ab}	89.28±3.5^{Abc}	81.83±4.20^{Ac}
	0.5 %	118.23±2.84^{Aa}	102.88±4.66^{Ab}	84.75±1.67^{Ac}	85.5±4.85^{Ac}
	1%	100.03±5.14^{Ba}	95.72±2.75^{Aa}	76.93±1.79^{Bb}	77.28±2.68^{Ab}
	1.5 %	88.22±3.64^{BCa}	85.26±5.42^{Aba}	73.50±2.76^{Bb}	66.28±4.45^{Bb}
	2%	76.90±3.84^{Ca}	72.04±4.36^{Ba}	59.93±3.56^{Cb}	47.47±2.35^{Cc}
VSL (µm/s)					
	Control	52.05±3.27^{Aa}	39.25±5.11^{Ab}	33.60±3.22^{b}	37.50±4.09^{Ab}
	0.5 %	59.18±2.48^{Aa}	40.24±3.76^{Ab}	34.33±4.66^{c}	39.92±1.90^{Abc}
	1%	42.60±3.66^{Ba}	31.78±2.03^{Abb}	26.38±1.76^{b}	33.28±4.00^{ABb}
	1.5 %	36.08±2.23^{Ba}	24.64±1.99^{Bb}	24.70±1.90^{b}	23.85±2.43^{Bb}
	2%	25.03±3.36^{Ca}	21.56±1.42^{Bb}	21.63±2.82^{b}	18.17±0.27^{Bb}
VAP (µm/s)					
	Control	67.12±3.01^{a}	51.12±4.51^{Ab}	43.95±3.16^{Ab}	45.57±3.52^{Ab}
	0.5 %	70.95±2.40^{a}	51.36±3.15^{Ab}	42.60±3.16^{Ac}	47.20±1.24^{Abc}
	1%	69.98±2.49^{a}	43.48±1.25^{Abb}	35.73±1.59^{Abb}	41.23±4.07^{ABb}
	1.5 %	45.96±3.12^{a}	35.38±2.33^{BCb}	33.53±1.55^{Bb}	31.87±2.18^{BCb}
	2%	36.4±2.70^{a}	30.7±1.58^{Cb}	29.47±2.41^{Bbc}	24.78±0.36^{Cc}
LIN (%)					
	Control	40.65±3.21^{AB}	38.35±4.85	37.42±2.73	46.03±5.02
	0.5 %	50.00±1.52^{A}	38.88±3.78	39.17±4.72	46.68±2.73
	1%	42.50±2.61^{AB}	33.68±2.91	34.30±2.14	42.82±4.31
	1.5 %	40.88±1.22^{AB}	29.00±2.01	33.82±2.81	36.07±3.48
	2%	33.13±2.99^{Bab}	29.90±0.73^{a}	35.70±3.42^{ab}	38.77±2.03^{b}
STR (%)					
	Control	77.7±2.15^{AB}	75.58±3.22	75.77±2.02	81.27±3.30
	0.5 %	83.32±1.09^{A}	77.02±3.00	76.50±4.02	69.82±12.05
	1%	78.63±1.94^{AB}	72.84±2.55	73.47±1.67	80.12±2.78
	1.5 %	79.08±0.97^{AB}	190.20±120.22	73.27±2.59	73.98±3.02
	2%	70.03±4.51^{B}	70.00±1.45	63.03±11.64	73.20±0.84
WOB (%)					
	Control	51.98±2.62^{AB}	49.12±4.43	49.08±2.43	55.92±4.10
	0.5 %	59.83±1.24^{Aa}	50.08±2.91^{ab}	49.43±3.24^{b}	56.10±3.43^{ab}
	1%	53.82±2.04^{AB}	45.68±2.34	46.52±1.89	53.02±4.12
	1.5 %	52.46±1.49^{ABa}	41.62±1.71^{b}	45.82±2.17^{ab}	48.47±3.35^{ab}
	2%	46.03±1.60^{Bab}	42.72±0.61^{a}	49.08±2.44^{ab}	52.90±2.47^{ab}
ALH (µm/s)					
	Control	4.90±0.30^{A}	4.28±0.22^{AB}	4.08±0.09^{A}	4.05±0.30^{A}
	0.5 %	4.27±0.12^{AB}	4.32±0.19^{A}	3.93±0.16^{A}	3.97±0.18^{A}
	1%	4.05±0.24^{B}	4.24±0.24^{AB}	4.05±0.07^{A}	3.68±0.14^{A}
	1.5 %	3.86±0.13^{B}	3.96±0.28^{AB}	3.62±0.08^{A}	3.47±0.21^{AB}
	2%	3.72±0.09^{Ba}	3.36±0.15^{Bb}	2.97±0.22^{Bc}	2.83±0.11^{Bc}
BCF (Hz)					
	Control	8.77±0.47^{A}	7.72±0.41^{AB}	7.50±0.27	8.05±0.56
	0.5 %	9.57±0.17^{Aa}	8.04±0.51^{Bab}	7.82±0.65^{b}	8.05±0.38^{b}
	1%	8.43±0.44^{A}	7.30±0.43^{AB}	7.45±0.44	7.87±0.52
	1.5 %	8.46±0.44^{Aa}	6.86±0.36^{ABab}	6.97±0.18^{ab}	7.03±0.45^{b}
	2%	6.02±0.77^{Ba}	6.28±0.26^{Aa}	6.95±0.66^{ab}	7.85±0.25^{b}

Velocity Straight Line (VSL), Velocity Curve Linear (VCL), Velocity Average Path (VAP), Wobble (WOB), Linearity (LIN), Straightness (STR), Amplitude of Lateral Head Displacement (ALH) and Beat Cross Frequency (BCF). A, B, C: indicates the difference between groups in the same row ($P < .05$). a, b, c, d: indicates the difference between times in the same column ($P < .05$).

Table 3. Mean oxidative stress levels (mean±sem) between experimental groups.

	MDA (nmol/ml)		GSH (nmol/ml)		GSH-Px (IU/g protein)		CAT(kU/g protein)	
	0. Hour	72. Hour	0. Hour	72. Hour	0. Hour	72. Hour	0. Hour	72. Hour
Control	11.18±0.42^{b}	11.58±0.65^{c}	0.70±0.55^{Aa}	1.38±0.81^{Ba}	7.70±0.32^{a}	7.80±0.34^{a}	176.51±12.48^{a}	193.74±16.92^{a}
0.5 %	8.26±1.26^{a}	8.54±0.79b^{a}	0.86±0.66^{Aba}	2.35±0.16^{Bb}	8.18±0.86^{a}	10.10±0.91^{ba}	194.48±22.74^{a}	263.73±31.39^{ba}
1%	8.52±0.79^{a}	7.31±0.37^{a}	1.06±0.70^{Acb}	2.36±0.14^{Bb}	9.26±0.45^{a}	9.73±0.62^{ba}	205.27±7.30^{a}	264.23±18.46^{ba}
1.5 %	7.53±1.45^{a}	6.94±0.26^{a}	1.1±0.61^{Adc}	2.81±0.11^{Bcb}	12.86±0.72^{b}	10.62±1.05^{b}	262.11±19.00^{b}	287.31±28.60^{b}
2%	9.15±1.2b^{a}	9.14±0.65^{b}	1.30±0.11^{Ad}	2.55±0.54^{Bcb}	8.39±0.71^{a}	9.02±0.54^{ba}	220.50±23.14^{ba}	225.70±39.90^{ba}

Superscript letters (a, b, c) indicate the difference between groups within the same time ($P < .001$). Superscript letters (A, B) shows the difference between times within the same group ($P < .05$).

Table 4. Mean abnormal sperm ratio (mean±sem) between experimental groups.

Groups	0. Hour	72. Hour
Control	7.16±0.98^{a}	3.66±0.42^{a}
0.5%	5.83±1.76^{a}	3.16±1.76^{a}
1%	10.00±2.26^{ab}	12.33±3.56^{b}
1.5%	14.00±2.00^{bc}	14.33±1.75^{b}
2%	16.67±2.00^{c}	20.16±4.40^{c}

Superscript letters (a, b, c) indicate the difference between groups ($P < .001$). There is no statistical difference between times within the same group ($P > .05$).

DISCUSSION

Since the freezing/thawing process of ram semen in sheep breeding causes serious damage to spermatozoa, chilled semen dilution method is used.[26] Cooling semen storage in rams also reduces sperm fertility efficiency, so the semen diluent medium should be supplemented with antioxidants and sperm motility should be increased for spermatozoa to reach the ampulla.[27] In the present study, the effects of APL added to semen diluent during cooling storage of semen on sperm kinematic parameters and oxidant status were investigated in rams.

Antioxidant compounds are used to prevent increased oxidative damages, different antioxidant substances added to the diluent during short-term storage of ram semen are reported to reduce increased LPO and also increase the reduced total antioxidant capacity induced by short time storage.[28,29] APL, a bee product, is a compound with high antioxidant activity.[30] APL has both androgenic activity [12] and a protective effect against toxic agents.[18] In addition mammalian spermatozoa contain a high polyunsaturated fatty acid ratio in the plasma membrane and a low cholesterol-to-phospholipid molar ratio.[31] These membranes are susceptible to peroxidation during aerobic long-term storage.[32] Storage of ram spermatozoa under cooling conditions can produce large amounts of ROS leading to loss of fertility.[29] It has been reported that an increase in oxidative stress may lead to a decrease in important sperm functions such as the acrosome reaction. [33] It is seen that different antioxidant compounds added to ram semen diluent have positive effects on semen quality.[28,34] A proper oxidant/antioxidant balance can improve sperm fertilization capacity.[35] In a study, it was reported that Chlorogenic Acid supplementation added to semen diluent decreased oxidative stress and increased progressive motility in short-term storage of semen in rams.[36] In the present study, progressive motility up to 72 hours was numerically superior in the group treated with 0.5% APL ($P < .05$). Antioxidants have an important role in preserving sperm physiological activities and can counteract the negative effects of the cooling process.[37] Because of its high polyphenol concentration, APL has excellent antioxidant activity.[6] MDA is a byproduct of lipid peroxidation that serves as an indicator of ROS-induced damage in spermatozoa.[38] In the present study, MDA level was found to be lower in all concentrations of APL compared to the control group. This indicates that APL leads to a decrease in MDA level by decreasing LPO. GSH, GSH-Px, and CAT are enzymatic and non-enzymatic antioxidant defense system parts.[39] GSH acts as a co-factor for GSH-Px, a protective enzyme that catalyzes the reduction of harmful H_2O_2 and other hydroperoxides.[40] Normally, semen contains antioxidants such taurine, catalase, glutathione, glutathione peroxidase (GSH-Px), and superoxide dismutase, which inhibit lipid peroxidation (LPO) and excessive ROS production.[41] Different antioxidant compounds are reported to protect sperm quality by increasing antioxidant activity in ram semen.[42] It has been reported that APL protects the semen from testicular toxicity due to its antioxidant properties.[18,43] In the present study, APL caused a increase in GSH level and GSH-Px and CAT activities in ram semen at all time periods.

Semen morphology is used as an important criterion in the evaluation of semen in domestic animals and it is stated

that semen with a high percentage of abnormalities reduces fertility.[44,45] The composition of semen diluents is reported to be effective in sperm abnormalities.[46] In the present study, the lowest abnormal sperm rates were observed in the 0.5% APL and control group at the 0th hour ($P < .001$). At high doses of APL, sperm quality decreased. This is thought to be due to the fact that it is above the physiological antioxidant level.

VCL, VSL and VAP are positively correlated with sperm motility, whereas VCL is highly correlated with sperm fertilization ability. It is reported that spermatozoa progression in cervical mucus is positively correlated with the VCL kinetic parameter.[47] In the present study, it was generally observed that VCL, VSL and VAP values decreased in all groups with the passage of time. In addition, higher VCL, VSL and VAP values were found in the control and 0.5% APL groups compared to the other experimental groups. This suggests that fertilization ability may be higher in control and 0.5% APL groups. In a study, it was reported that antioxidant compounds improved motility and kinematic parameters in ram semen. This is reported to be due to the energy production that occurs as a result of the antioxidant compound used affecting aerobic respiration.[48]

High concentrations of antioxidant compounds can damage spermatozoa and cause a decrease in fertility rate.[49] In the present study, the high abnormal sperm rates in the 1%, 1.5% and 2% APL groups support the literature. In another study, it was reported that gallic acid added to ram semen diluent increased the rate of abnormal semen as the hours progressed.[25] In the present study, it was observed that the rate of abnormal spermatozoa increased as time progressed in all groups except the 0.5 APL group. It is thought that this may be due to the change in the osmotic pressure of the semen diluent of the antioxidant compound used.[50] Moreover abnormal semen is formed during spermatogenesis. It is also thought that abnormalities other than spermatogenesis may be due to osmotic changes in the semen diluent.

As a result the use of APL at doses between 0.5% and 1.5% added to semen diluent for short-term storage of semen in rams has been proven to improve the quality of ram semen for up to 72 hours.

Ethics Committee Approval: Consent was obtained from Fırat University Animal Experiments Local Ethics Committee (Date: 16.11.2022, Number of Sessions: 2022/18-08).

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Protective Effect of Tiger Nut (*Cyperus Esculentus*) Against Monosodium Glutamate-Induced Reproductive Dysfunction in Male Wistar Rats

Yer Bademi (*Cyperus esculentus*)'nin Erkek Wistar Sıçanlarda Monosodyum Glutamat Kaynaklı Üreme Fonksiyon Bozukluğuna Karşı Koruyucu Etkisi

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ABSTRACT

Monosodium glutamate (MSG) is a widespread flavour enhancer linked to health risks, including male reproductive dysfunction. This study investigated tiger nut (*Cyperus esculentus*) as a potential protective agent against MSG-induced reproductive issues in male Wistar rats. Forty adult rats were divided into four groups: control, MSG-only (2 mg/g), tiger nut-only (500 mg/kg), and MSG+tiger nut combination (2 mg/g MSG + 500 mg/kg tiger nut). Treatments were administered orally for 28 days, with analyses conducted at days 14 and 28. Results showed significant variations in sperm parameters. At 14 days, the tiger nut group showed highest sperm motility (88.60±4.04%) and count (100.60±3.21×10^6/mL), while MSG reduced sperm viability (70.00±4.69%). By 28 days, MSG significantly decreased sperm motility (41.80±4.92%) and viability (54.80±6.76%). MSG increased sperm abnormalities at 14 days (13.60±2.51%) but normalized by 28 days. The MSG+tiger nut combination eliminated certain sperm abnormalities like coiled tail and tail-without-head. Gonadometric parameters remained stable throughout the study, indicating tiger nut's ability to maintain testicular architecture despite MSG exposure. Initial body weight increases in the MSG group normalized by weeks 3-4. The study concludes that tiger nut juice significantly protects against MSG-induced low sperm quality in male Wistar rats, suggesting its potential as a protective supplement for populations with unavoidable MSG exposure. Future research should explore long-term effects and cellular mechanisms.

Keywords: *Cyperus esculentus*, fertility, monosodium glutamate, rat, spermatotoxicity.

ÖZ

Monosodyum glutamat (MSG), erkek üreme fonksiyon bozukluğu da dahil olmak üzere sağlık riskleriyle ilişkilendirilen yaygın bir lezzet arttırıcıdır. Bu çalışmada, yer bademinin (*Cyperus esculentus*) erkek Wistar sıçanlarında MSG kaynaklı üreme sorunlarına karşı potansiyel koruyucu etkisi araştırılmıştır. Kırk yetişkin sıçan, kontrol, MSG (2 mg/g), yer bademi (500 mg/kg) ve MSG + yer bademi kombinasyonu (2 mg/g MSG + 500 mg/kg yer bademi) olmak üzere dört gruba ayrılmıştır. Uygulamalar 28 gün boyunca ağız yoluyla gerçekleştirilmiş; analizler 14. ve 28. günlerde yapılmıştır. Sonuçlar sperm parametrelerinde önemli değişiklikler olduğunu göstermiştir. 14. günde, yer bademi grubu en yüksek sperm hareketliliği (%88,60±4,04) ve sayısı (100,60±3,21×10^6/mL) gösterirken, MSG uygulaması sperm canlılığını (%70,00±4,69) azaltmıştır. MSG, 28. günde sperm hareketliliğini (%41,80±4,92) ve canlılığını (%54,80±6,76) önemli ölçüde azaltmıştır. MSG, 14. günde sperm anormalliklerini artırmış (%13,60±2,51); ancak 28. günde normale dönmüştür. MSG+yer bademi kombinasyonu ise kıvrık kuyruk ve başsız kuyruk gibi bazı sperm anormalliklerini ortadan kaldırmıştır. Gonadometrik parametreler çalışma boyunca sabit kalmış, bu da yer bademinin MSG maruziyetine rağmen testis yapısını koruma yeteneğini göstermektedir. MSG grubundaki başlangıçtaki vücut ağırlığı artışları, 3-4. haftalarda normale dönmüştür. Bu çalışma, yer bademi suyunun erkek Wistar sıçanlarında MSG kaynaklı düşük sperm kalitesine karşı önemli ölçüde koruma sağladığı sonucuna vararak, kaçınılmaz MSG maruziyeti olan popülasyonlar için koruyucu bir takviye olarak potansiyelini ortaya koymaktadır. Gelecekteki araştırmalar uzun vadeli etkileri ve hücresel mekanizmaları araştırmalıdır.

Anahtar Kelimeler: *Cyperus Esculentus*, fertilite, monosodyum glutamat, sıçan, spermatotoksisite

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INTRODUCTION

Monosodium glutamate (MSG) is a widely used flavour enhancer, particularly prevalent in oriental cuisine and processed foods worldwide.[1] As a sodium salt of glutamic acid, it provides the distinctive umami taste that enhances food palatability.[2] Despite its Generally Recognized as Safe (GRAS) status by food safety regulatory agencies, mounting evidence suggests potential health risks associated with MSG consumption, including obesity, metabolic disorders, and notably, reproductive dysfunction.[3] Of particular concern are studies linking MSG to male reproductive health issues, including oligozoospermia, abnormal sperm morphology, and testicular damage.[4,5]

The search for protective agents against MSG-induced reproductive toxicity has gained importance, especially given MSG's ubiquity in modern diets. Tiger nut (*Cyperus esculentus*), a nutrient-rich tuber, has emerged as a promising candidate due to its unique nutritional profile.[6] Rich in fibre, proteins, essential fatty acids, vitamins C and E, and various minerals, tiger nut has traditionally been consumed in various forms, including raw, roasted, or as a beverage called "Horchata".[7,8] While its nutritional benefits are increasingly recognized, tiger nut's potential protective effects against reproductive toxicants remain largely unexplored.

Previous studies have examined either MSG's adverse effects on male reproduction or tiger nut's general health benefits separately. MSG has been shown to cause testicular damage through oxidative stress and inflammatory pathways.[9-11] While tiger nut has demonstrated antioxidant and anti-inflammatory properties.[12] However, the potential protective role of tiger nut against MSG-induced reproductive dysfunction represents a novel area of investigation. This research gap is particularly significant given the increasing exposure to MSG in modern diets and the need for practical, accessible protective measures.

Our study uniquely investigates the concurrent administration of MSG and tiger nut, examining whether tiger nut's nutritional and antioxidant properties could mitigate MSG-induced reproductive dysfunction in male Wistar rats. This approach differs from previous research by evaluating the protective rather than merely therapeutic potential of tiger nut, focusing on practical, readily measurable reproductive parameters, and examining the interaction between MSG and tiger nut in a controlled experimental setting. We hypothesized that tiger nut supplementation protects against MSG-induced reproductive dysfunction by improving sperm parameters and maintaining normal gonadometric measures. This investigation aims to provide practical insights for populations with unavoidable MSG exposure and potential applications in animal breeding programs.

MATERIALS AND METHODS

Experimental Animals

This study was carried out according to the regulations and principles that govern both care and use of experimental animals for research purposes by the Animal Care and Use Ethics Committee at the University of Ibadan (Date: 16/5/2018, Approval No: UI-ACUREC/17/0069). Forty adults male Wistar rats (150 ± 10g) were housed under controlled conditions (35-36ºC, 50 ± 15% humidity, 12:12 light-dark cycle). Animals were monitored daily for signs of stress or adverse reactions, including changes in feeding behaviour, activity levels, and coat condition. No unexpected events or adverse reactions were observed during the study period.

Dosage Selection and Treatment

The MSG dosage (2 mg/g) was established through a weight-based calculation accounting for average daily dietary MSG exposure scaled to rat metabolism. This dose represents a moderate exposure level that allows for observation of potential reproductive effects while remaining within physiologically relevant bounds for food additive consumption. The tiger nut dose (500 mg/kg) was selected based on the measured nutrient content of tiger nut juice, specifically its antioxidant components and bioactive compounds, to provide sufficient protective capacity while maintaining safe consumption levels for daily administration. Both doses were determined to be within the practical oral administration range for rats of this size and age. Treatments were administered orally for 28 days, with careful monitoring of administration technique to ensure complete dosing. The 28-day duration was chosen to span at least two complete cycles of spermatogenesis in rats, allowing for observation of potential effects on sperm production and maturation.

Materials and Preparation

MSG (98%) and tiger nut were sourced from Bodija Market, Ibadan, Nigeria (7.3775ºN, 3.9470ºE). All other chemicals used in the study were of the highest available grade in Nigeria. The tiger nut juice was prepared by crushing the tubers, followed by manual extraction of the juice (milk) using a 0.075 mm plastic sieve.

Study Design

The 40 adult male Albino rats (Wistar strain) were randomly grouped into four (n=10). Group A was the

Control and received 0.5ml of distilled water orally. Group B, received oral MSG at 2 mg/g body weight orally, Group C received oral tiger nut at 500 mg/kg body weight while Group D received a combination of MSG at 2 mg/g and tiger nut at 500 mg/kg body weight. All treatments were administered for 28 days. Five rats from each group were humanely sacrificed 24 hours after both 14 and 28 days of treatment. Following the sacrifice, the testes and epididymis were harvested, and semen samples were collected for analysis. Additionally, gonadometric assessments were conducted.

Gonadometric Analysis

The testes were detached from the epididymis as outlined by Ajani and Omoyeni.[12] Both the testes and epididymis were weighed using an Electronic Weight Scale (Ohaus Corporation, USA), while the length and diameter of the testes were determined using an Electronic Veiner Caliper (Mitutoyo Corporation, Japan) following the methodology outlined by Ajani and Omoyeni.[12]

Semen Analysis

Semen samples were collected from the left caudal epididymis using a surgical incision method as described by Oyeyemi and Fayomi.[13] Briefly, the epididymis was carefully excised and placed in a pre-warmed (37°C) petri dish containing 1 mL of physiological saline (0.9% NaCl). Multiple incisions were made in the cauda epididymis using a sterile surgical blade (size 11) to release the spermatozoa.

For morphological assessment, a drop of semen was placed on a clean, pre-warmed (37°C) glass slide and stained using Wells and Awa staining method (Wells and Awa stain). The stained slides were examined under a light microscope at 400× magnification to evaluate sperm morphology.

Sperm viability was assessed using the eosin-nigrosin staining technique. The staining solution was prepared using 5% eosin Y and 10% nigrosin in distilled water. One drop of semen was mixed with two drops of the stain on a pre-warmed slide, and a thin smear was prepared. After air-drying, the slides were examined under a light microscope at 400× magnification. Live spermatozoa remained unstained (white), while dead spermatozoa appeared pink.

For sperm motility assessment, 2-3 drops of semen were mixed with an equal volume of pre-warmed (37°C) 2.9% sodium citrate buffer (pH 7.4) on a clean glass slide as described by Zemjanis.[14] The preparation was immediately examined under a light microscope at 400× magnification using a pre-warmed (37°C) stage. The percentage of motile sperm was calculated by counting both motile and immotile spermatozoa in several microscopic fields, evaluating a minimum of 200 sperm cells.

Statistical Analysis

The Shapiro–Wilk test was done and it showed that the parameters were Gaussian. Data were expressed as mean ± SD and analysed using One-Way ANOVA, followed by the post-hoc Tukey's test to evaluate significant differences within the groups. GraphPad Prism version 9.0 (GraphPad Software, San Diego, California, USA) was used for the statistics analysis. Value of $P \leq .05$ was considered significant.

RESULTS

Body Weight Changes in Response to MSG Treatment Over a Four-Week Period

The initial body weights showed no significant differences ($P > .05$) between all groups. In week one and two, the MSG-only group showed significantly higher body weights (179.57±12.45g and 182.58±17.64g respectively, $P < .05$) compared to other groups. However, by weeks three and four, there were no significant differences ($P > .05$) in body weights across all groups, suggesting that the initial weight gain effect of MSG normalized over time (Table 1).

Table 1. Body Weight of the Male Wistar Rats in Different treatment groups across experiment duration

Body Weight (g)	A Control	B MSG only	C Tiger nut only	D MSG & Tiger nut
Initial Weight	147.56 ± 4.63	147.75 ± 16.52	155.8 ± 2.61	146.1 ± 3.15
Week One	157.83 ± 9.07[a]	179.57 ± 12.45[b]	160.79 ± 10.60[a]	162.16 ± 5.90[a]
Week Two	155.78 ± 11.07[a]	182.58 ± 17.64[b]	161.65 ± 11.33[a]	163.76 ± 9.96[a]
Week Three	185.48 ± 18.27	203.93 ± 17.42	197.17 ± 7.29	201.92 ± 11.06
Week Four	203.52 ± 19.42	214.76 ± 20.30	204.24 ± 14.27	205.68 ± 9.10

MSG: Monosodium glutamate, Values expressed as Means ± Standard Deviation (SD), [a,b:]Means with different superscripts within row are significantly different ($P < .05$).

Effects of Tiger Nut and MSG on Sperm Characteristics and Morphology at 14 and 28 Days Post-Treatment

The tiger nut only group showed significantly higher sperm motility (88.60±4.04%, $P < .05$) compared to other groups. Sperm viability was significantly lower in the MSG-only group (70.00±4.69%, $P < .05$). Sperm count was significantly highest in the tiger nut group (100.60±3.21×106 sperm cells/mL, $P < .05$) and lowest in the MSG-only group (57.00±23.82×106 sperm cells/mL, $P < 0.05$) (Table 2).

Table 2. Semen characteristics of the male Wistar rats in different treatment groups at 14 days post-treatment

Parameters	A (Control)	B (MSG only)	C (Tiger nut only)	D (MSG + Tiger nut)
Sperm Motility (%)	75.00±12.25^{a}	59.40±11.15^{b}	88.60±4.04^{c}	71.40±8.20^{a}
Sperm viability (%)	81.20±11.43^{ab}	70.00±4.69^{c}	89.20±5.26^{a}	72.20±11.43^{bc}
Sperm count x106 sperm cells/mL	83.40±6.35^{a}	57.00±23.82^{b}	100.60±3.21^{c}	80.00±9.87^{a}

MSG: Monosodium glutamate, Values expressed as Means ± Standard Deviation (SD), ^{a,b,c}:Means with different superscripts within row are significantly different ($P < .05$).

After 28 days, the MSG-only group showed significantly reduced sperm motility (41.80±4.92%, $P < .05$) and viability (54.80±6.76%, $P < .05$) compared to other groups. The tiger nut group maintained the highest values for these parameters. Sperm count was significantly lower in the MSG-only group (51.80±10.64×10^{6} sperm cells/mL, $P < .05$) compared to other groups (Table 3).

Table 3. Semen characteristics of the male Wistar rats in different treatment groups at 28 days post-treatment

Parameters	A (Control)	B (MSG only)	C (Tiger nut only)	D (MSG + Tiger nut)
Sperm Motility (%)	80.80±7.46a	41.80±4.92^{b}	92.40±3.36^{a}	79.40±7.80^{a}
Sperm viability (%)	78.40±12.66a	54.80±6.76^{b}	91.80±3.83^{a}	82.20±5.40^{a}
Sperm count x10^{6} sperm cells/mL	78.80±8.67a	51.80±10.64^{b}	100.20±14.8^{a}	87.80±14.29^{a}

MSG: Monosodium glutamate, Values expressed as Means ± Standard Deviation (SD), ^{a,b}:Means with different superscripts within row are significantly different ($P < 0.05$).

The MSG-only group showed significantly higher total abnormal cells (13.60±2.51%, $P < .05$) and percentage abnormality (3.14±0.38%, $P < .05$) compared to other groups. Individual morphological abnormalities showed no significant differences ($P > .05$) between groups. The total cells counted were comparable across all groups ($P > .05$) (Table 4).

Table 4. Sperm morphology of the male Wistar rats in different treatment groups at 14 days post-treatment

Parameters (%)	A (Control)	B (MSG only)	C (Tiger nut only)	D (MSG + Tiger nut)
Abnormal head	2.00±0.00	2.60±0.89	2.50±0.58	3.00±0.00
Coiled tail	1.67±0.58	3.00±0.00	2.00±0.00	1.00±0.00
Bent tail	2.00±0.82	3.30±1.26	2.50±0.58	2.25±0.00
Head without tail	2.00±0.00	2.33±0.58	2.00±0.00	2.75±0.96
Tail without head	3.33±0.58	2.67±1.15	1.00±0.00	2.00±1.41
Looped tail	2.00±0.00	3.00±1.00	2.00±0.82	2.00±1.41
Rudimentary tail	2.25±0.50	2.33±1.53	0.00±0.00	0.00±0.00
Double head	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Curved mid-piece	2.00±0.00	2.50±0.71	2.67±1.52	1.00±0.00
Total abnormal cells	8.00±1.00^{a}	13.60±2.51^{b}	8.60±1.82^{a}	9.75±1.26^{a}
Total cells counted	422.20±11.43	435.20±38.69	463.60±47.22	427.75±10.69
Percentage abnormality	1.90±0.28^{a}	3.14±0.38^{b}	1.86±0.54^{a}	2.28±0.26^{a}

MSG: Monosodium glutamate, Values expressed as Means ± Standard Deviation (SD), ^{a,b}:Means with different superscripts within row are significantly different ($P < .05$).

Table 5 examined sperm morphology across four groups (Control, MSG only, Tiger nut only, and MSG + Tiger nut) over 28 days. The MSG + Tiger nut combination eliminated coiled tail and tail-without-head abnormalities ($P < .05$), but also showed the lowest total sperm count (429.50 ± 19.09) and highest overall abnormality rate (2.20 ± 0.42%). The

Tiger nut only group demonstrated the lowest percentage of total abnormalities (1.66 ± 0.56%), while most other sperm parameters showed no significant differences between groups.

Gonadometric Assessment Following MSG and Tiger Nut Treatment at 14 and 28 Days Post-Treatment

No significant differences ($P > .05$) were observed in any gonadometric parameters (testicular weights, lengths, diameters, and epididymal weights) between groups after 14 days of treatment (Table 6).

Similar to the 14-day results, no significant differences ($P > .05$) were observed in any gonadometric parameters between groups after 28 days of treatment, suggesting that neither MSG nor tiger nut significantly affected testicular size and epididymal weight (Table 7).

Table 5. Sperm morphology of the male Wistar rats in different treatment groups at 28 days post-treatment

Parameters (%)	Control	MSG only	Tiger nut only	MSG + Tiger nut
Abnormal head	2.75 ± 0.96	2.25 ± 0.50	3.00 ± 0.00	2.00 ± 0.00
Coiled tail	3.00 ± 0.00[a]	2.00 ± 1.00[ab]	2.00 ± 0.00[ab]	0.00 ± 0.00[b]
Bent tail	2.00 ± 0.00	3.00 ± 0.00	2.50 ± 0.71	2.00 ± 0.00
Head without tail	2.00 ± 0.00	1.67 ± 0.57	2.00 ± 0.00	1.50 ± 0.71
Tail without head	2.00 ± 0.00[a]	2.33 ± 1.15[a]	2.50 ± 0.71[a]	0.00 ± 0.00[b]
Looped tail	2.33 ± 0.58	2.00 ± 0.00	2.33 ± 0.58	2.00 ± 0.00
Rudimentary tail	2.00 ± 1.41	2.50 ± 0.71	2.50 ± 0.71	3.00 ± 0.00
Double head	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Curved mid-piece	1.00 ± 0.00[b]	2.25 ± 0.96[a]	2.00 ± 1.41[ab]	1.50 ± 0.71[ab]
Total abnormal cells	9.75 ± 1.50	9.60 ± 1.14	7.80 ± 2.95	9.50 ± 2.12
Total cells counted	510.50 ± 62.67[a]	491.40 ± 27.80[a]	470.0 ± 53.61[a]	429.50 ± 19.09[b]
Percentage abnormality	1.93 ± 0.35[ab]	1.98 ± 0.16[ab]	1.66 ± 0.56[b]	2.20 ± 0.42[a]

MSG: Monosodium glutamate, Values expressed as Means ± Standard Deviation (SD), [a,b]:Means with different superscripts within row are significantly different ($P < .05$).

Table 6. Gonadometric assessment of the male Wistar rats in different treatment groups at 14 days post-treatment

Parameters	A Control	B MSG only	C Tiger nut only	D(MSG ± Tiger nut)
LTW (g)	1.01±0.09	1.11±0.15	1.10±0.06	1.11±0.08
RTW(g)	1.06±0.08	1.08±0.16	1.04±0.10	1.11±0.07
LTL (mm)	18.20±1.22	18.44±0.97	18.48±0.19	18.08±0.41
RTL (mm)	18.56±0.54	18.18±1.06	17.98±0.74	17.40±0.71
LTD (mm)	10.34±0.82	10.40±0.26	10.72±0.69	10.42±0.08
RTD (mm)	11.16±0.88	10.38±0.52	10.64±0.67	10.46±0.53
Ep. Wt. (g)	0.35±0.18	0.30±0.06	0.29±0.07	0.39±0.06

MSG: Monosodium glutamate, LTW: left testes weight, RTW: right testes weight, LTL: left testes length, RTL: right testes length, LTD: left testes diameter, RTD: right testes diameter, Ep. Wt.: Epididymal Weight, Values expressed as Means ± Standard Deviation (SD).

Table 7. Gonadometric assessment of the male Wistar rats in different treatment groups at 28 days post-treatment

Parameters	A Control distilled water for 28 days	B MSG only for 28 days	C Tiger nut only	D (MSG + Tiger nut)
LTW (g)	1.09±0.17	1.11±0.14	1.17±0.11	1.21±0.10
RTW(g)	1.12±0.16	1.13±0.15	1.21±0.06	1.24±0.07
LTL (mm)	18.12±1.13	18.76±0.83	19.24±0.84	19.76±0.46
RTL (mm)	18.20±1.10	18.56±0.95	19.24±0.49	19.58±0.82
LTD (mm)	10.60±0.25	11.10±0.72	11.28±0.61	11.18±0.70
RTD (mm)	10.72±0.73	11.10±0.66	11.38±0.30	11.42±0.40
Ep. Wt. (g)	0.47±0.10	0.44±0.09	0.41±0.05	0.52±0.06

MSG: Monosodium glutamate, LTW: left testes weight, RTW: right testes weight, LTL: left testes length, RTL: right testes length, LTD: left testes diameter, RTD: right testes diameter, Ep. Wt.: Epididymal Weight, Values expressed as Means ± Standard Deviation (SD).

DISCUSSION

Our study reveals significant insights into the protective effects of tiger nut against MSG-induced reproductive dysfunction in male Wistar rats. The findings contribute to both the understanding of MSG's reproductive toxicity and the potential protective role of natural supplements in maintaining reproductive health.

The observed initial increase in body weight following MSG treatment aligns with established literature on MSG's orexigenic effects.[15–17] This finding supports previous research demonstrating MSG's potential role in obesity development through altered feeding patterns and metabolic changes. However, the normalization of body weight by weeks 3-4 suggests potential adaptation mechanisms that warrant further investigation.

A key finding of our study is the marked improvement in sperm parameters following tiger nut administration. The significant enhancement in sperm motility, viability, and count in the tiger nut-treated groups extends beyond previous findings by Ekaluo et al., [18] who reported only motility improvements. Our comprehensive analysis demonstrates tiger nut's broader positive impact on overall semen quality, suggesting multiple mechanisms of action that could include enhanced antioxidant protection through vitamin C and E content, improved cellular membrane integrity from essential fatty acids, and potential hormonal modulation through bioactive compounds.[19,20]

The protective effect of tiger nut against MSG-induced spermatotoxicity represents a novel finding. While MSG administration alone resulted in significant reductions in sperm motility and count, concurrent tiger nut supplementation maintained these parameters near control levels. This protective effect was particularly evident in sperm morphology, where tiger nut treatment significantly reduced the incidence of abnormal forms typically associated with MSG exposure. These findings build upon previous work by Nosseir et al.[21] and Onakewhor et al., [22] suggesting tiger nut's potential role in maintaining normal sperm development and maturation.

Regarding gonadometric parameters, the absence of significant changes between groups requires careful interpretation. Rather than indicating a lack of effect, this finding suggests that tiger nut supplementation may help maintain normal testicular architecture even in the presence of MSG exposure.[23-25] This maintenance of normal gonadometric parameters, combined with improved sperm quality, indicates that tiger nut's protective effects may operate through biochemical and cellular mechanisms that preserve functional capacity without necessarily altering gross anatomical measures.

The practical implications of our findings are significant, particularly for population groups with high MSG exposure through dietary habits, animal breeding programs where reproductive efficiency is crucial, and the development of natural protective supplements for reproductive health. While our study provides valuable insights, we acknowledge several limitations that suggest directions for future research. The 28-day treatment period, while sufficient to demonstrate acute effects, could be extended to cover complete spermatogenic cycles. Inclusion of histological analysis would provide cellular-level insights into protective mechanisms. Hormonal profiling would help elucidate potential endocrine-mediated effects. Dose-response studies could optimize protective effects. These limitations do not diminish the significance of our findings but rather highlight opportunities for more comprehensive future investigations.

As a result, our findings establish a foundation for understanding tiger nut's protective potential against reproductive toxicants and suggest practical applications in both human and animal reproductive health. Tiger nut demonstrates significant promise as a natural protective agent against MSG-induced reproductive dysfunction. The improvement in sperm parameters and maintenance of normal gonadometric measures support its potential use as a supplement in contexts where MSG exposure is concerning. These findings contribute to the growing body of evidence supporting natural dietary interventions in reproductive health maintenance and suggest practical applications in both human and animal reproductive health management.

Ethics Committee Approval: This study was carried out according to the regulations and principles that govern both care and use of experimental animals for research purposes by the Animal Care and Use Ethics Committee at the University of Ibadan (Approval No: UI-ACUREC/17/0069), with strict adherence to guidelines regarding the well-being of the animals, such as proper housing, standard feeding, humane handling and disease prevention and control (Date: 16/5/2018).

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Indicator Microflora of Ducks and Chickens in Home Farm Conditions

Küçük Ölçekli Çiftlik Koşullarında Ördek ve Tavukların İndikatör Mikroflorası

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ABSTRACT

The aim of this study was to determine the risks of the circulation of zoonotic bacteria in poultry in homesteads. We selected for the study litter samples (10 samples each) of Muscovy ducks and chickens (Hisex breed) aged 100-110 days. The samples were examined using certified nutrient media and equipment in accordance with international standards: ISO 6887-1:2017; ISO 21528-1:2017; ISO 11290-1:2017; ISO 10273:2017; ISO 6579-1:2017; ISO/FDIS 7218; and DSTU 8534:2015. Litter samples from clinically healthy ducks and chickens were examined for the detection of potentially pathogenic bacteria of the Enterobacteriaceae family, *Listeria spp., Enterococcus spp., Pseudomonas aeruginosa*. In the studied biomaterial, representatives of *Klebsiella spp., Yersinia spp., Salmonella spp., Pseudomonas aeruginosa, Listeria spp*. were not detected. The content of Escherichia coli ($5.0x10^5$ CFU/g and $6.7x10^6$ CFU/g) and Enterococcus faecalis ($2.4x10^8$ CFU/g and $1.2x10^8$ CFU/g), respectively, in chicken and duck litter samples is considered physiological. Bacteriological examination of the droppings of clinically healthy chickens and Muscovy ducks, raised on a free-range homestead revealed no carriers of pathogenic bacteria, indicating that there are no possible risks of unchecked zoonotic pathogen spread from the consumption of "backyard" poultry products. *Escherichia coli* and *Enterococcus faecalis* in litter samples are considered to be physiological.

Keywords: Chicken, ducks, housing conditions, microflora

ÖZ

Bu çalışmanın amacı, küçük ölçekli çiftlik koşullarında kanatlılarda zoonotik bakterilerin yayılma risklerini belirlemektir. Çalışma için 100-110 günlük yaştaki Muscovy ördekleri ve tavukların (Hisex cinsi) altlık örnekleri (her birinden 10 örnek) seçilmiştir. Örnekler, uluslararası standartlara uygun olarak sertifikalı besin ortamları ve ekipmanlar kullanılarak incelenmiştir: ISO 6887-1:2017; ISO 21528-1:2017; ISO 11290-1:2017; ISO 10273:2017; ISO 6579-1:2017; ISO/FDIS 7218; ve DSTU 8534:2015. Klinik olarak sağlıklı ördek ve tavuklardan alınan altlık örnekleri *Enterobacteriaceae* familyası, *Listeria spp., Enterococcus spp., Pseudomonas aeruginosa* gibi potansiyel patojenik bakterilerin tespiti için incelenmiştir. İncelenen biyomateryalde, *Klebsiella spp., Yersinia spp., Salmonella spp., Pseudomonas aeruginosa, Listeria spp*. gibi bakteriler tespit edilmemiştir. Tavuk ve ördek altlık örneklerinde sırasıyla *Escherichia coli* ($5.0x10^5$ CFU/g ve $6.7x10^6$ CFU/g) ve *Enterococcus faecalis* ($2.4x10^8$ CFU/g ve $1.2x10^8$ CFU/g) içerikleri, fizyolojik olarak kabul edilmektedir. Serbest dolaşımlı küçük ölçekli bir çiftlikte yetiştirilen klinik olarak sağlıklı tavukların ve Muscovy ördeklerinin dışkılarının bakteriyolojik incelemesinde patojenik bakteri taşıyıcısı görülmemiştir; bu da "bahçe tavukçuluğu" kümes hayvanı ürünlerinin tüketiminden kaynaklanan kontrolsüz zoonotik patojen yayılımı riskinin bulunmadığını göstermektedir. Altlık örneklerindeki *Escherichia coli* ve *Enterococcus faecalis*'in, fizyolojik olduğu düşünülmektedir.

Anahtar Kelimeler: Tavuk, ördek, barınma koşulları, mikroflora

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INTRODUCTION

Modernising its economy with the goals of enhancing citizen welfare, preserving biological diversity, transitioning to a green economy, and making Europe climate-neutral is the plan for the growth of the modern European Union (Official website of the European Union, 2024).[1] The so-called "European Green Deal" aims to achieve the aforementioned approach. In this regard, fresh perspectives on animal husbandry in affluent nations are gained. The shifts in opinions towards backyard chicken production are the most evident. These days, poultry owners raise their birds for uses other than their personal eating, giving them a family-like care and deepening their emotional ties to them.[2]

There is relatively little information on the demographics of backyard owners and on the traits, upkeep, and welfare of herds because backyard animals are frequently privately held and the goods they produce are usually not marketed.[2,3]

According to the authors, the average flock size in France in recent years was five laying hens, and the majority of owners retained exclusively laying hens (78.4%).[4] In 86.6% of cases, the owners either routinely or occasionally donated eggs to their family members. Contacts with other poultry owners were common (68.9%), and the application of bioprotection techniques was subpar. Keeping domestic animals (53.2%), processing (72.4%), and egg consumption (93.3%) were the primary reasons for having chicken flocks. The necessity of evaluating health hazards in order to enhance their management is emphasised.

In the US, keeping small flocks of chickens for their eggs, meat, and maybe company is becoming a more and more common pastime. Such private backyard flocks often include domestic chickens (*Gallus gallus, forma domestica*), turkeys (*Meleagris gallopavo, forma domestica*), and *Anatidae* birds, such as ducks, geese, and swans. According to the authors, this common pastime also puts the owners' health at risk because of the high zoonotic potential of bacterial, viral, fungal, and parasitic illnesses that harm poultry.[5-9] Because home chicken farming is one of the fastest-growing sectors in the world, other writers draw attention to the necessity for legislative consolidation and the application of biosecurity principles. [10,11] Although the authors concentrate on the potential for backyard chickens to spread pathogens to wild birds, the threat posed by domestic chickens to wild birds has historically been understated, which supports the need for legislative regulation and the introduction of bioprotection principles (bioisolation and bioretention).

The findings of a study on the prevalence of bacterial and viral infections circulating in small flocks of non-commercial chicken in the Canadian province of Ontario are highlighted by Brochu et al., 2019.[12] According to the authors, bacterial agents such as *Mycoplasma synoviae, Campylobacter spp., Salmonella spp., Brachyspira spp*., and *Mycoplasma gallisepticum* were found in (37%, 36%, 35%, 23%, and 3%) of farms that were surveyed. In addition, influenza virus A H10N8 (low pathogenic) was isolated from the turkey, and among viral pathogens, infectious bronchitis virus, avian adenovirus, infectious laryngotracheitis virus, avian reovirus, and infectious bursal disease virus were found in (39%, 35%, 15%, 4%, and 1%) of cases, respectively. The significant rise in these non-commercial poultry flocks, the dearth of knowledge on zoonotic pathogens in these flocks, the elevated danger of new pathogen reservoirs as a result of poor biosecurity procedures, and the restricted availability of veterinary care all served as catalysts for the study.[12]

Laying hens were the most common species of birds (93.4%), followed by ducks and geese (35.3%), turkeys (33.8%) and grill chickens (33.1%), according to the authors' analysis of the structure of domestic poultry populations in the Canadian province of Alberta. Additionally, (58.1%) of owners reported that they were primarily new to production (73.1% kept birds for less than 5 years, 25.6% kept birds for less than 1 year); many kept multiple species, and the majority did not separate flocks based on species or purpose (81.8% - personal consumption, 48.2% - sale of eggs); accordingly, the owners reported inconsistent use of medical measures (vaccination, treatment, veterinary consultation).[13] In recent years, it has been reported that the environment is an important factor influencing gut microbiota.[14]

While the FAO emphasises intensive, sub-intensive, and extensive poultry farming systems, the backyard poultry industry falls into the fourth category of poultry farming, which has the lowest level of biosecurity, because backyard plot owners are typically unaware of the precautions that should be taken to protect their flock from infectious diseases and limit their transmission.[15] At the same time, a number of bacterial and viral pathogens, including *Campylobacter, Salmonella, Escherichia, Mycoplasma*, and others, pose a hazard to domestic chicken flocks. These pathogens include Newcastle disease, Marek disease, Infectious Bronchitis, Gumboro (IBD) disease, Infectious Laryngotracheitis, and Avian Influenza.[16-18] Additionally, there is a significant chance that poultry owners will come into direct contact with recognised zoonotic infections. One issue that puts the public's health at danger is the spread of infectious illnesses in poultry.

Due to their restricted access to veterinary care, poor biosecurity procedures, and higher danger of coming into touch with wild birds, small poultry flocks might operate as reservoirs for obligate avian and zoonotic infections. However, little is known about the incidence of zoonotic infections in flocks of non-commercial poultry, despite the possible hazards.[19-21]

The aforementioned thus supports the need for a thorough investigation into the epizootic status of poultry flocks housed on homesteads, the species composition of pathogenic and opportunistic microflora circulating among poultry of various species within homestead flocks, the specificity of the species composition of the digestive tract microbiome in various bird species, etc.

This study aimed to determine the possible risks of zoonotic pathogen transmission by comparing the composition of indicator bacteria in the droppings of clinically healthy ducks and chickens housed as a small flock of domestic poultry in a homestead in the Kyiv region.

MATERIALS AND METHODS

Animal Groups and Sampling

Fecal samples were collected from clinically healthy 100-110 day-old Muscovy ducks and Hisex-bred chickens housed in a homestead in a private village in the Kyiv region. The birds were allowed to roam freely and had free access to water. They were fed twice a day with chopped and steamed wheat and maize along with kitchen scraps. Samples of droppings were collected according to the State Standard of Ukraine 8703-1:2017. "Diagnostic for infectious disease. Part 1. Methods for collection, packaging and transport of samples", individually from the cloaca using a sterile swab. The swabs were placed in tubes with transport medium. (Ethics Date: 26/11/2024, Ethics decision no: 022.2024)

Bacteriological Studies

Tubes with samples (10 samples each from chickens and musk ducks) in a thermal container (temperature 2-80C) in a transport environment were delivered to the scientific laboratory of the Faculty of Veterinary Medicine and further processed in accordance with: a) Preparation of test samples, initial suspension and tenfold dilutions for microbiological examination was carried out in accordance with ISO 6887-1:2017; b) Isolation and determination of the most probable number of *Enterobacteria, E. coli, Klebsiella spp.*, was carried out by ISO 21528-1:2017; c) Isolation and identification of Yersinia enterocolitica was carried out in accordance with ISO 10273:2017; d) Isolation and determination of the most probable number (MPN test) of *Enterococcus* was carried out in accordance with DSTU 8534:2015; e) Isolation and identification of *Listeria spp./Listeria monocytogenes* was carried out in accordance with ISO 11290-1:2017; f) Isolation and identification of Salmonella spp., carried out by ISO 6579-1:2017; g) Isolation and determination of Pseudomonas aeruginosa was carried out in accordance with the "Methodological recommendations. Detection and identification of Pseudomonas aeruginosa in environmental objects (food products, water, wastewater)".

Statistical Analysis

The MPN test was used to estimate the number of viable cells of a particular microorganism.

RESULTS

Litter samples taken from clinically healthy ducks and chickens kept in the conditions of a small flock of domestic poultry were studied to identify potentially pathogenic bacteria of the *Enterobacteriaceae* family *(Salmonella spp., Escherichia coli, Yersinia spp., Klebsiella spp*.), as well as *Listeria spp./Listeria monocytogenes, Pseudomonas aeruginosa* and *Enterococcus spp.*

It should be noted that in the studied biomaterial of microorganisms – the representatives of *Klebsiella spp., Yersinia spp., Salmonella spp., Pseudomonas aeruginosa, Listeria spp*. were not detected.

As a result of bacteriological studies of droppings from ducks and chickens, 20 cultures of the *Enterobacteriaceae* family were isolated (gram-negative motile rods, catalase-positive, and oxidase-negative; the isolated cultures were facultative anaerobes that ferment glucose with the formation of acid and gas).

The cultures under study are motile gram-negative rods based on their morphology. One-day-old cultures produced homogeneous turbidity in a liquid medium, specifically meat peptone broth (MPB), along with a tiny amount of white amorphous material that readily disintegrated when shaken. Bismuth-sulfite agar, a selective differential diagnostic medium, did not support the growth of the isolates under study.

Cultures developed S-shaped colonies that were clear, fragile, and greyish on meat peptone agar (MPA) medium. These colonies had a diameter of 2-4 mm. On the selective differential diagnostic medium xylose-lysine deoxycholate agar (XLD), *Escherichia coli* cultures developed as yellow

colonies; the surrounding medium's colour changed from red to yellow. On the *Salmonella* M1078/Sereda Raj Hans differential diagnostic media, *Escherichia coli* cells developed blue colonies. Isolated cultures developed into green colonies on the chromogenic medium HiCrome *E. coli* Agar M 12951, which is used for *Escherichia coli* detection and counting. *Escherichia coli* cultures digested lactose and glucose, producing gas and acid; they did not convert nitrates to nitrites or release H2S; instead, they created indole instead of urea. Thus, the 20 isolates matched *Escherichia coli* based on their enzymatic and cultural-morphological characteristics.

Bacteriological analyses of dropping samples from ducks and hens resulted in the isolation of 20 *Enterococcus spp*. cultures in addition to *Escherichia*. Gram-positive cocci-like non-motile microorganisms, facultative anaerobes, catalase- and oxidase-negative, and fermenting glucose with the formation of acid without gas, the isolated cultures formed black colonies with a diameter of up to 1.5 mm on bile esculin agar with sodium azide (Bile esculinazid agar, manufactured by the company Sanimed-M), encircled by a brown-black zone of altered medium colour. The M1830HiCrome VREAgar medium produced blue-green colonies. The 20 isolates that were chosen matched the traits of *Enterococcus faecalis* based on their morphological, cultural, and enzymatic traits.

Consequently, the following were separated and identified from the sample under study: Ten *Enterococcus faecalis* and ten *Escherichia coli* cultures were isolated and identified from chickens, while ten *Escherichia coli* and ten *Enterococcus faecalis* cultures were isolated and identified from ducks.

The MPN indication (MPN test) was used to evaluate the quantitative content of the isolated bacteria. *Escherichia coli* and *Enterococcus faecalis* were chosen as indicator bacteria, and their MPN.

The most likely amount of *Escherichia coli* in the chicken dropping samples that were analysed (Table 1) was found to be between $4.6x10^2$ and $4.6x10^6$ colony-forming units (CFU) in 1 g of the sample; at a 95% probability level, the actual number of germs in 1 g was between $9.0x10^1$ and $1.96x10^7$ CFU/g. The analysed chicken dropping samples had an average of $5.0x10^5$ CFU/g of *Escherichia coli*. With the actual number of microorganisms in 1 g at a 95% probability level falling between $2.0x10^3$ and $>1.1x10^9$ CFU/g, the MPN of *Enterococcus faecalis* in the investigated chicken droppings samples was found to be between $2.0x10^3$ and $>1.1x10^9$ CFU/g. In the chicken dropping samples under study, the average value of the MPN indicator *Enterococcus faecalis* was 2.4x108 CFU/g (Table 1).

There was a difference in the number ratio of *Enterococcus faecalis* and *Escherichia coli* in the studied samples of chicken droppings: in 9 out of 10 tested samples, the value of *Enterococcus faecalis* MPN exceeded the *Escherichia coli* MPN by 1-5 lg, while the exceedance of *Enterococcus faecalis* MPN by 1 lg was registered in two samples (samples No. 5 and 9); an excess of MPN *Enterococcus faecalis* by 2 lg was registered in one sample (sample No. 7); an excess of MPN *Enterococcus faecalis* by 3 lg was registered in 3 samples (samples No. 1, 3, 8); an excess of MPN *Enterococcus faecalis* by 4 lg was registered in 3 samples (samples No. 4, 6, 10).

Table 1. The most likely number of indicator bacteria in chicken droppings samples

	Indexes			
	Escherichia coli, (1)CFU		*Enterococcus faecalis* (1)CFU	
Samples, №	*Escherichia coli* content, (2) MPN in 1.0 g	The actual number of microorganisms in 1 g at a 95% level of probability is within:	*Enterococcus faecalis* content, (2) MPN in 1.0 g	The actual number of microorganisms in 1 g at a 95% level of probability is within:
1	$1.1x10^3$	$2.0x10^2$ - $4.0x10^3$	$1.1x10^6$	$2.0x10^5$-$4.0x10^6$
2	$2.4x10^4$	$4.0x10^3$-$9.9x10^4$	$1.1x10^4$	$2.0x10^3$-$4.0 x10^4$
3	$4.6x10^2$	$9.0x10^1$-$1.96x10^3$	$1.1x10^5$	$2.0x10^4$-$4.0x10^5$
4	$1.1x10^5$	$2.0x10^4$-$4.0x10^5$	$>1.1x10^9$	--
5	$1.1x10^5$	$2.0x10^4$-$4.0x10^5$	$1.1x10^6$	$2.0x10^5$-$4.0x10^6$
6	$4.6x10^4$	$9.0x10^3$-$1.96 x10^5$	$1.1x10^8$	$2.0x10^7$-$4.0x10^8$
7	$4.6x10^3$	$9.0x10^2$-$1.96x10^4$	$4.6x10^5$	$9.0x10^4$-$1.96x10^6$
8	$4.6x10^6$	$9.0x10^5$-$1.96x10^7$	$> 1.1x10^9$	--
9	$4.6x10^4$	$9.0x10^3$-$1.96 x10^5$	$4.6x10^5$	$9.0x10^4$-$1.96x10^6$
10	$1.1x10^3$	$2.0x10^2$-$4.0 x10^3$	$4.6x10^7$	$9.0x10^6$-$1.96x10^8$
min-max	$4.6x10^2$-$4.6x10^6$	$9.0x10^1$-$1.96x10^7$	$1.1x10^4$->$1.1x10^9$	$2.0x10^3$>$1.1x10^9$
Average value	$5.0x10^5$	--	$2.4x10^8$	

Notes: (1) CFU – colony-forming units; (2) MPN - the most probable number.

The MPN of *Escherichia coli* in the examined samples of duck droppings (table 2) was recorded in the range of 1.1x104 - 2.4x10^7 CFU in 1 g of the sample (with the actual number of microorganisms in 1 g at the 95% probability level within 2.0x10^3 - 9.9x10^7 CFU/g). The average value of MPN *Escherichia coli* in the studied samples of duck droppings was 6.7x10^6 CFU/g.

In the duck droppings samples that were analysed (table 2), the MPN of *Escherichia coli* was found to be between 1.1x10^4 and 2.4x10^7 CFU in 1 g of the sample, while the actual number of microorganisms in 1 g at the 95% probability level was between 2.0x10^3 and 9.9x10^7 CFU/g. The average MPN *Escherichia coli* value in the duck dropping samples under study was 6.7x10^6 CFU/g. *Enterococcus faecalis*'s MPN in the duck dropping samples under study ranged from 1.1x10^3 to 4.6x10^8 CFU in 1 g of the sample; at a 95% confidence level, the actual number of germs in 1 g was between 2.0x10^2-1.96x10^9 CFU/g. In the duck dropping samples that were analysed, the average MPN *Enterococcus faecalis* value was 1.2x10^8 CFU/g (Table 2).

The following ratios of the numbers of *Enterococcus faecalis* and *Escherichia coli* were recorded in the studied samples of duck droppings in 5 out of 10 tested samples, while MPN *Enterococcus faecalis* exceeded MPN *Escherichia coli* by 1-2 lg. At the same time, an excess of MPN *Enterococcus faecalis* by 1 lg was registered in one sample (sample No. 1); an excess of MPN *Enterococcus faecalis* by 2 lg was registered in 4 samples (samples No. 3, 4, 6, 7). An excess of MPN *Escherichia coli* by 1 lg was registered in 4 samples (samples No. 1, 5, 8, 10). In sample 9 MPN of *Escherichia coli* and *Enterococcus faecalis* was within 1.1x10^7.

Comparing the MPN of *Escherichia coli* in the examined samples of droppings from chickens and ducks, the following was established: the average value of the MPN of *Escherichia coli* in chickens was 5.0x10^5 CFU/g; in ducks, this indicator was 6.7x10^6 CFU/g, which exceeded the similar value in chickens by 1 lg.

Table 2. The most likely number of indicator bacteria in duck droppings samples

	Indexes			
	Escherichia coli, (1) CFU		Enterococcus spp., (1) CFU	
Samples, №	*Escherichia coli* content, (2) MPN in 1.0 g	Actual number of microorganisms in 1 g at a 95% level of probability is within the:	*Enterococcus faecalis* content, (2) MPN in 1.0 g	The actual number of microorganisms in 1 g at a 95% level of probability is within:
1	1.1x10^6	2.0x10^5-4.0x10^6	4.6x10^7	9.0x10^6-1.96x10^8
2	1.1x10^6	2.0x10^5-4.0x10^6	1.5x10^7	3.0x10^6-3.8x10^7
3	1.1x10^6	2.0x10^5-4.0x10^6	4.6x10^8	9.0x10^7-1.96x10^9
4	1.1x10^6	2.0x10^5-4.0x10^6	4.6x10^8	9.0x10^7-1.96x10^9
5	1.1x10^4	2.0x10^3-4.0 x10^4	1.1x10^3	2.0x10^2-4.0 x10^3
6	2.4x10^6	4.0x10^5-9.9 x10^6	1.1x10^8	2.0x10^7-4.0x10^8
7	1.1x10^6	2.0x10^5-4.0x10^6	1.1x10^8	2.0x10^7-4.0x10^8
8	2.4x10^7	4.0x10^6-9.9x10^7	1.1x10^6	2.0x10^5-4.0x10^6
9	1.1x10^7	2.0x10^6-4.0x10^7	1.1x10^7	2.0x10^6-4.0x10^7
10	2.4x107	4.0x10^6-9.9 x10^7	1.1x10^6	2.0x10^5-4.0x10^6
min-max	1.1x10^4-2.4x10^7	2.0x10^3-9.9 x10^7	1.1x10^3 4.6x10^8	2.0x10^2-1.96x10^9
Average value	6.7x10^6	--	1.2x10^8	--

Notes: (1) CFU – colony-forming units; (2) MPN - the most probable number.

The average value of MPN *Enterococcus faecalis* in the studied samples of chicken droppings was 2.4x10^8 CFU/g; in ducks, this indicator was 1.2x10^8 CFU/g.

DISCUSSION

According to research by Muyyarikkandy et al.[22], the dynamic state of the microbiome ensures that healthy chickens have a certain level of resistance to diseases. Furthermore, the bird's microbiome contributes to the synthesis of nutrients, influences the development of the immune system, and so on, all of which contribute to the bird's overall physiological development and well-being. Environmental factors and the conditions under which birds are kept (well-being) are linked to the microbiome's condition. Research on how biotic and abiotic factors affect the microbiome of chickens housed in small flocks in the "backyard" is still important because it can be used to

predict the likelihood of zoonotic disease and infectious poultry disease outbreaks, boost biosecurity, and guarantee sufficient productivity.[23]

Since the dynamics of the content of indicator organisms in a particular ecological/biological niche may indicate the presence of certain pathogens, it is possible to use microorganisms, such as bacteria, fungi, viruses, and bacteriophages, to assess the risks of zoonotic disease outbreaks among populations. It is also important to monitor the state and/or changes in the composition of indicator biomarkers.[24-28]

The purpose of the study was to evaluate the composition of microorganisms in the droppings of clinically healthy chickens and musk ducks housed in a small herd on a homestead in order to ascertain the possible risks of zoonotic pathogen transmission to poultry owners (product consumers). Identification of potentially harmful bacteria, including *Listeria monocytogenes, Yersinia spp., Salmonella spp., Klebsiella spp., Pseudomonas aeruginosa, Escherichia coli, and Enterococcus spp*., was the goal of bacteriological investigations.

It is significant to highlight that no pathogenic bacteria (*Yersinia, Salmonella, Pseudomonas aeruginosa, Listeria,* or *Klebsiella spp.)* were identified from clinically healthy fowl housed in the "backyard" in this investigation. The examined litter samples contained *Enterococcus faecalis* and *Escherichia coli*. This investigation did not isolate and identify other kinds of bacteria that might be found in the litter, specifically *Bifidobacterium spp., Lactobacillus spp*., etc.

Using the test for identifying the MPN of microorganisms, which not only allowed for the detection of certain bacterial genera but also the estimation of their number, the evaluation of chosen indicator bacteria was conducted.[29] The average MPN for *Escherichia coli* in the examined samples of duck and chicken droppings was $6.7x10^6$ CFU/g and $5.0x10^5$ CFU/g, respectively, according to the analysis of the results of bacteriological investigations. In chickens and ducks, the corresponding indication for *Enterococcus faecalis* was $2.4x10^8$ CFU/g and $1.2x10^8$ CFU/g.

The microbiome composition (as measured by indicator bacteria) of clinically healthy ducks and hens kept in a small backyard flock was thus described in the pilot study's initial findings. It was shown that there were no carriers of harmful bacteria, which means there were no possible dangers of zoonotic outbreaks due to the consumption of poultry products and/or the unchecked spread of zoonotic agents. A level of 10^5–10^8 CFU/g of indicator bacteria (*Escherichia coli* and *Enterococcus faecalis*) in litter samples is regarded as normal.

One of the predominant kinds of obligatory intestinal microflora, or microorganisms, in the distal portions of animals' intestines is *Escherichia coli*. Gram-positive lactic acid bacteria, such as *Enterococcus faecalis*, are often found in soil, surface water, and plants as part of the digestive tracts of animals of different species.[30,31]

As the authors note, however, there has been a lot of recent discussion about the benefits and drawbacks of *Enterococcus faecalis*. On the one hand, the bacteria are utilised as probiotics, starters, and biological control agents to enhance human or animal health, [32] while on the other hand, members of this species are known to cause nosocomial infections. However, the authors assert that strain-specific virulence factors in enterococci exist.[33-35]

The data pertaining to the condition of the microbiome of poultry under various conditions of keeping (in industrial enterprises and small flocks in the "backyard") partially align with the results we obtained regarding the content of indicator bacteria in the droppings of clinically healthy chickens and musk ducks kept in a free-range private sector homestead.[36] The scientists specifically reported that whereas Bacteroides, which was linked to better chicken development performance, was more common in home poultry, the concentration of zoonotic Campylobacter in poultry rose during industrial maintenance. According to another study, free-range poultry in the US had a 33% lower rate of circulating antimicrobial-resistant Salmonella than poultry kept in industrial facilities.[37]

Therefore, the goal of our study was to ascertain the possible hazards of zoonotic infections spreading among a small flock of ducks and chickens in a farmhouse in the Kyiv region. At the same time, we did not find any evidence of zoonotic pathogen circulation among the species under investigation. The information gathered is crucial for a more thorough comprehension of the connections in the chain within the "One-Health" concept—the environment, human health, and animal health. The findings will advance our understanding of backyard chicken production and the microbiome of fowl. Our next research will focus on identifying any viruses that may be circulating in this herd that are harmful to birds and, maybe, humans.

In summary as a results bacteriological analysis of the droppings of clinically healthy musk ducks and chickens raised on free range in a homestead revealed no signs of pathogenic bacterial carriage, and thus no danger of

unchecked zoonotic pathogen spread from consuming "backyard" poultry products. *Enterococcus faecalis* and *Escherichia coli*, two indicator bacteria, were thought to be physiologically present in litter samples.

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The Investigation of Sexual and Species Dimorphism in the Foot of Chukar Partridge (*Alectoris chukar*) and Gray Partridge (*Perdix perdix*) Using Geometric Morphometric and Symmetric Analyses

Kınalı Keklik (*Alectoris chukar*) ve Çil Keklik (*Perdix perdix*) Ayaklarındaki Cinsiyet ve Tür Dimorfizminin Geometrik Morfometrik ve Simetrik Analizler Kullanılarak İncelenmesi

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ABSTRACT

Morphological differences can provide insights into species' ecological adaptations and evolutionary processes. This study focuses on examining the effects of sex and species dimorphism on foot morphology in two different partridge species, the Chukar Partridge (*Alectoris chukar*) and the Gray Partridge (*Perdix perdix*). A total of 68-foot samples, including both right and left foot, were analyzed from 34 partridges collected in Sivas province. The analysis revealed that, regardless of sex, the first toe of Chukar Partridges was longer than that of Gray Partridges. When comparing species, the angle between the toes of female Chukar Partridges was wider than that of female Gray Partridges, while the angle between the toes of male Gray Partridges was wider than that of male Chukar Partridges. In terms of sex differences, the angle between the toes of male Gray Partridges was wider than that of female Gray Partridges, while the angle between the toes of female Chukar Partridges was wider than that of male Chukar Partridges. The contribution of directional asymmetry to variation was found to be lower than that of fluctuating asymmetry in both shape and size, suggesting that the asymmetry may result from developmental differences rather than lateral bias. Overall, the width of the toe angle was linked to habitat preferences and ecological adaptations. These findings suggest that the foot morphology of partridges may be shaped by factors such as sex, species, and habitat, and that these adaptations may help birds cope with environmental challenges.

Keywords: Chukar partridge, gray partridge, geometric morphometry, morphology, symmetry analysis

ÖZ

Şekil farklılıkları, türlerin ekolojik adaptasyonları ve evrimsel süreçleri hakkında bilgi sağlayabilir. Çalışmanın odak noktası, iki farklı keklik türü olan Kınalı Keklik (*Alectoris chukar*) ve Çil Kekliklerin (*Perdix perdix*) cinsiyet ve ırk dimorfizminin ayak morfolojisine etkilerini incelemektir. Sivas ilinden toplanan 34 keklikten sağ ve sol olmak üzere toplam 68 ayak örneği analiz edildi. Analiz sonuçlarına göre cinsiyet fark etmeksizin kınalı kekliklerin baş parmağı çil kekliklerden uzundu. Irklar kıyaslandığında dişi kınalı kekliğin parmakları arasındaki açı dişi çil keklikten genişti. Erkek çil kekliğin parmakları arasındaki açı ise erkek kınalı keklikten daha genişti. Cinsiyetler kıyaslandığında ise erkek çil kekliklerin parmakları arasındaki açı dişi çil kekliklerden, dişi kınalı kekliklerin parmakları arasındaki açı erkek kınalı kekliklerden daha genişti. Directional asimetrinin varyasyona katkısı, şekil ve boyut üzerindeki fluctuating asimetriden daha düşüktü. Bu da asimetrinin, taraf farkından ziyade gelişimsel farklılıklardan oluştuğunu göstermektedir. Sonuç olarak parmaklar arasındaki açının genişliği habitat tercihleri ve ekolojik adaptasyonlarla ilişkilendirildi. Kekliklerin ayak morfolojisinin cinsiyet, ırk ve yaşam alanı gibi faktörlerle şekillenebileceğini ve bu adaptasyonların kuşların çevresel zorluklarla başa çıkmasına yardım ettiği düşünülmektedir.

Anahtar Kelimeler: Çil keklik, kınalı keklik, geometrik morfometri, morfoloji, simetri analizi

INTRODUCTION

When examining evolutionary processes, morphological traits undergo variations in shape and size due to the strong influence of biological factors, and these variations play a significant role in species identification. While the dimensions of morphometric traits may be similar, species can often be distinguished by their shapes.[1-3] In recent years, there has been a noticeable increase in the use of geometric morphometric analyses in studies of bird species.[4-6,25-30] Morphometric analysis focuses on the dimensions of materials, whereas geometric morphometric analysis concentrates on shape. The use of geometric morphometric analysis is advantageous over traditional size analyses, as shape is a more decisive factor and tends to exhibit greater variation within species groups than size. [7,8,31-34] Since form and function are closely linked to species' ecology, body traits can provide insight into how animals forage for food or evade predators.[9,10]

The fundamental principle of geometric morphometric analyses is the examination of shapes using Cartesian coordinates based on anatomical landmarks.[11] Various ecological and biological processes can lead to shape differences between species and within-species groups, which may also result from factors such as disease, geographic adaptation, ontogenetic development, or evolutionary divergence.[12] The Chukar Partridge (*Alectoris chukar*), a ground-dwelling bird specialized in running and walking, matures early. Its locomotor performance is influenced by factors such as age, sex, species, and breeds.[13,14] The Gray Partridge (*Perdix perdix*), on the other hand, nests on the ground and inhabits open agricultural fields and grasslands. Once common in agricultural landscapes, the Gray Partridge has experienced a significant population decline over the past fifty years, necessitating its classification for conservation purposes.[15]

Partridges have strong, short, and sturdy feet, which help them move easily in steep terrains. Being anisodactyl, they have a total of four toes, three facing forward and one facing backward. The forward-facing toes are separated and strong, enhancing grip on the ground. The hind toe (hallux) is usually small and makes minimal or no contact with the ground. The toes end in strong claws, which are used for digging, gripping, and defense.[36]

In this study, we aim to investigate the effects of sex and species dimorphism on the foot morphology of two wild partridge species, the Chukar Partridge and the Gray Partridge, using geometric morphometric analysis. Additionally, we aim to analyse symmetry-asymmetry patterns to assess differences between the left and right foot.

MATERIALS AND METHODS

Animal

In the province of Sivas, a total of 34 partridges were utilized in this study, comprising 22 Chukar Partridges (*Alectoris chukar*) (15 males and 7 females) and 12 Gray Partridges (*Perdix perdix*) (6 males and 6 females) harvested by hunters from the Gürlevik and Yılanlı General Hunting Grounds in Hafik District, the Gaziköy and Uçuk General Hunting Grounds in Şarkışla, and the Aşağı Boğazkesen General Hunting Ground in Sivas Center. Partridges are all similar-aged wild birds that have hatched in the wild. The average weight of male Chukar Partridges was 582 g, while females weighed 458 g; for Gray Partridges, males weighed 358 g and females 311 g. The foot of the partridges taken from the hunters were immediately photographed without any processing. For the geometric morphometric analysis, the left foot of each partridge was used, while the right and left foot were utilized for symmetry-asymmetry analysis. The foot was photographed from a dorsal view at a 90-degree angle from the Os tarsometatarsus, positioned at 31 cm for analysis. Ethical approval for the study was obtained from the Ethics Committee of Selçuk University Faculty of Veterinary Medicine (Date: 05/09/2024, Approval No: 2024/139).

Geometric Morphometric and Symmetric Analysis

To facilitate the analysis of the photographs, it was necessary to convert them into Tps format. This conversion was performed using TpsUtil (version 1.74). Landmark coordinates were established by placing landmarks on the photographs converted to Tps format using the tpsDig (version 2.30) program. A total of nine landmarks were utilized (Figure 1). The dataset containing the coordinates was uploaded to Morphoj (version 1.07a) for geometric morphometric analysis and symmetry-asymmetry analysis. The landmarks used are as follows:[20]

Lm1: Lateral of the articulatio metatarsophalangea I
Lm2: Phalanx terminalis digiti I
Lm3: Medial of the articulatio metatarsophalangea I
Lm4: Phalanx terminalis digiti II
Lm5: Tela interdigitalis intermedia
Lm6: Phalanx terminalis digiti III
Lm7: Tela interdigitalis lateralis
Lm8: Phalanx terminalis digiti IV
Lm9: Lateral of the articulatio metatarsophalangea IV

Statistical Analysis

For geometric morphometric analysis, regression analysis and Principal Component Analysis (PCA) were conducted using the MorphoJ software. The PCA analysis determined

the direction and magnitude of change in the principal components relative to the total shape variation from the mean shape. Discriminant Function Analysis (DFA) was performed to identify group characteristics based on sex and species.[20] Additionally, symmetry-asymmetry analysis was conducted in MorphoJ to determine the symmetric and asymmetric characteristics of the right and left foot.[35]

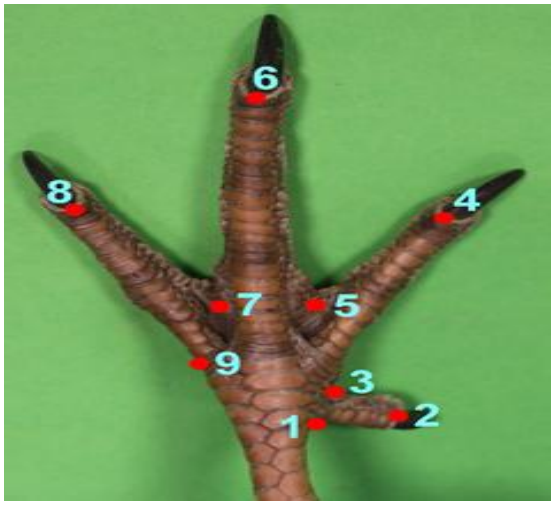

Figure 1. Representation of landmarks; Lm1: Lateral of the articulatio metatarsophalangea I, Lm2: Phalanx terminalis digiti I, Lm3: Medial of the articulatio metatarsophalangea I, Lm4: Phalanx terminalis digiti II, Lm5: Tela interdigitalis intermedia, Lm6: Phalanx terminalis digiti III, Lm7: Tela interdigitalis lateralis, Lm8: Phalanx terminalis digiti IV, Lm9: Lateral of the articulatio metatarsophalangea IV.

RESULTS

Prior to conducting Principal Component Analysis (PCA), regression analysis was performed. The regression analysis conducted on the shape centroid size (PCs) indicated that species accounted for 4.09% of the shape variation (P = .2900). Based on this finding, it was determined that the foot shape variations concerning the species factor addressed in this study were not size-dependent. Consequently, no statistically significant allometric component was identified.

According to the results of the Principal Component Analysis, the first principal component (PC1) explained 31.40% of the total shape variation, while the cumulative contribution of the first two principal components (PC1 + PC2) reached 60.19% of the total shape variation. A significant inflection point among the principal components was particularly observed between PC2 and PC3.

Figure 2 presents wireframe graphics obtained from PCA, illustrating the direction and extent of variation related to PC1 and PC2 in terms of total shape variation. The regions where shape differences associated with PC1 and PC2 are concentrated include the terminal phalanges of the first (Lm2), second (Lm4), third (Lm6), and fourth (Lm8) toes. The most pronounced changes in the mean shape according to PC1 are a reduction in the angle between the third and fourth toes and an increase in the angle of the first toe. In contrast, the most significant changes observed in PC2 involve an increase in the angles between the second and third toes, as well as between the third and fourth toes.

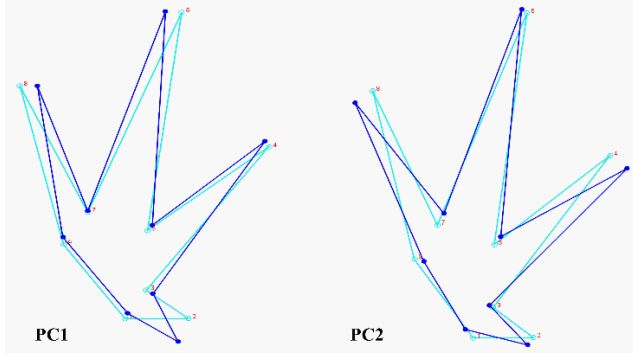

Figure 2. Wireframe graphical representation of shape differences concerning PC1 and PC2. Dark blue represents the positive bounds of principal component scores.

The discriminant function analysis (DFA) compared the foot morphology of the partridges in relation to gender and species factors. Figure 3A depicts the comparison of the foot morphology of the chukar partridge, while Figure 3B illustrates that of the gray partridge, with a focus on the gender factor. According to the analysis, the angle between the second and third toes of female gray partridges is narrower compared to males. Conversely, the angle between the second and third toes of female chukar partridges is wider than that of males.

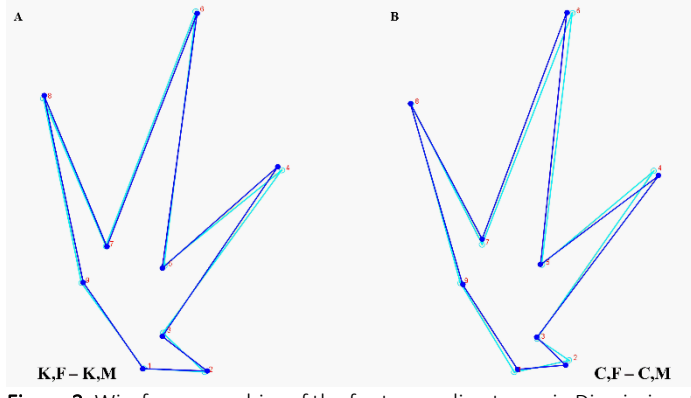

Figure 3. Wireframe graphics of the foot according to sex in Discriminant Function Analysis (K: Chukar partridge, C: Gray partridge, F: Female, M: Male). In Figure 3A, the light blue color represents the female Chukar partridge, while the dark blue color represents the male Chukar partridge; in Figure 3B, the light blue color represents the female Gray partridge, and the dark blue color represents the male Gray partridge.

The DFA graphs presented in Figure 4 illustrate the comparison of partridge foot morphology based on species factors. In Figure 4A, the angle between the second and third toes of female gray partridges is narrower than that of female chukar partridges, while the angle between the third and fourth toes is wider in female gray partridges compared to females of the chukar species. Additionally, the hallux of female chukar partridges is longer than that of female gray partridges. Figure 4B shows that the angles between the second and third, as well as the third and fourth toes of male gray partridges, are wider compared to those of male chukar partridges. Furthermore, the hallux of male chukar partridges is longer than that of male gray partridges.

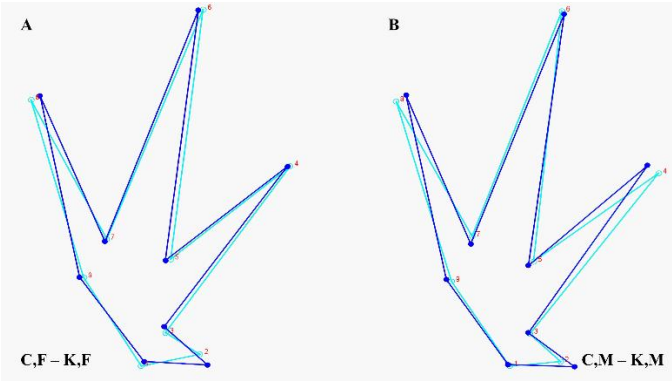

Figure 4. Wireframe graphics of the foot according to breed in Discriminant Function Analysis (K: Chukar partridge, C: Gray partridge, F: Female, M: Male). In Figure 4A, the light blue color represents the female Gray partridge, while the dark blue color represents the female Chukar partridge; in Figure 4B, the light blue color represents the male Gray partridge, and the dark blue color represents the male Chukar partridge.

The Procrustes ANOVA analysis indicated that measurement error was not significant. Variation among individuals in terms of "Shape" and "Size" was statistically significant ($P < .0001$). While fluctuating asymmetry for "Size" was statistically significant ($P < .0001$), directional asymmetry was not significant ($P = .9057$). The symmetric component was statistically significant ($P < .0001$). Regarding "Shape," both symmetric ($P < .0001$) and asymmetric components (fluctuating ($P < .0001$) and directional ($P = .0005$)) were statistically significant. The variation rates of differences among individuals were 97.42% for "Size" and 51.87% for "Shape" (Table 1). The contribution of directional asymmetry (DA) to variation was lower than that of fluctuating asymmetry regarding shape and size, suggesting that asymmetry is primarily due to developmental differences rather than side differences. Nevertheless, it was observed that the partridge foot included in the dataset exhibited a tendency in usage, resulting in a statistically significant value for directional asymmetry in terms of shape (Figure 5).

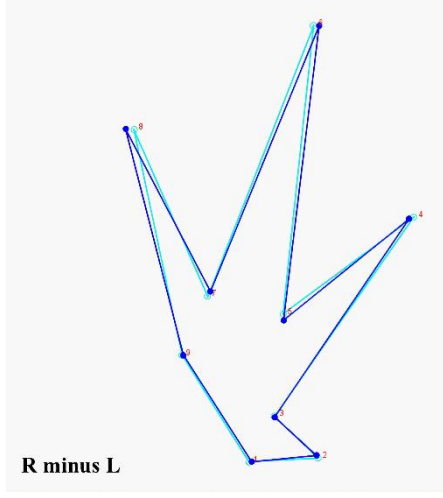

Figure 5. Symmetry-asymmetry graph. Dark blue represents the left foot.

Table 1. Morphological variation in foot size and shape calculated by Procrustes ANOVA.

Size	% Variance Explained	SS	MS	dF	F	*P*
Individual	97.425	343212.290249	16343.442393	21	45.91	<.0001
Side	0.001	5.112705	5.112705	1	0.01	.9057
Ind*Side	2.121	7475.255787	355.964561	21	16.83	<.0001
Error 1 (Imaging)	0.264	930.610306	21.150234	44	1.41	.0864
Error 2 (Digitizing)	0.187	660.234013	15.005318	44	0.83	.7491
Shape	% Variance Explained	SS	MS	dF	F	*P*
Individual	51.873	0.51334933	0.0017460862	294	1.66	<.0001
Side	4.252	0.04208750	0.0030062498	14	2.86	.0005
Ind*Side	31.179	0.30855610	0.0010495105	294	9.89	<.0001
Error 1 (Imaging)	6.605	0.06537211	0.0001061236	616	1.06	.1817
Error 2 (Digitizing)	6.200	0.06135782	0,0000996069	616	0.97	.6780

SS: sums of squares; MS: mean squares; dF: degrees of freedom, F: statistics and parametric p values are provided for each effect.

DISCUSSION

Natural habitats, foraging behaviour, ecological niche, and factors such as genetic variation significantly influence the development of foot morphology.[16] This study aimed to investigate whether the factors of species and sex contribute to shape variation in the foot of two distinct partridge species, based on the hypothesis that "the variation in the foot of species inhabiting similar geographical regions with comparable foraging conditions of the same sex would be limited." The findings indicate that the different environmental conditions experienced by male and female chukar partridges inhabiting rocky, steep, and rugged terrains, as well as male and female gray partridges residing in fields, flat landscapes, and pastures, result in phenotypic variation arising from adaptations influenced by these distinct living conditions and sexual dimorphism.

Geometric morphometric analyses revealed that, regardless of sex, the hallux of the gray partridge is shorter than that of the chukar partridge. Chukar partridges inhabit rocky and rugged terrains,[17] which necessitates a longer hallux for improved grip and stability. A longer hallux enables these partridges to effectively navigate steep slopes and rocky surfaces while foraging. Additionally, it enhances their ability to probe the ground for seeds and insects, thereby increasing their chances of survival. Conversely, gray partridges, typically found in more open and flat fields,[18] possess a shorter hallux that suffices for food searching in these relatively flat environments, minimizing competition with grasses. Furthermore, an increase in toe length provides a broader surface area for weight distribution, enhancing maneuverability in steep and uneven terrains. In summary, toe length may reflect various ecological adaptations and habitat preferences. Similarly, the angle between the toes is largely related to these factors. In our study, the angle between the toes of female gray partridges was found to be narrower than that of female chukar partridges. A wider toe angle enhances stability and grip, which is a critical adaptation for chukar partridges living in rocky and uneven environments. This adaptation facilitates a more balanced distribution of weight on steep inclines and challenging terrains, thereby improving stability and gripping ability. Moreover, the angle between the toes influences the bird's maneuverability while foraging, allowing for quick directional changes necessary for evading predators. A narrower toe angle in gray partridges renders them more adept at running and making rapid maneuvers in flat and pasture-like environments. Consequently, it is hypothesized that the toe angle may serve as an adaptable trait that can be developed in response to varying environmental contexts and locomotion requirements.

Another notable finding from our study is that the angle between the toes of female gray partridges is narrower than that of male gray partridges. Male partridges possess greater body weight and longer, stronger legs compared to females. The balanced distribution of body weight across the foot and toes is critical for stability and grip. It is posited that a wider angle between the toes may facilitate this weight distribution. One of the adaptations associated with sexual dimorphism in partridges may be the differences in toe angles. Interestingly, it was found that the angle between the toes of female chukar partridges is wider than that of male chukar partridges. Furthermore, male gray partridges exhibit a greater angle between their toes compared to male chukar partridges. The relatively shorter femur and more streamlined body structure of gray partridges, as well as the rounder body shape characteristic of chukar partridges adapted to rugged terrains, suggest that the influence of species may play a role in these sexual adaptations. These morphological variations may be a response to the ecological demands and habitat preferences specific to each species and sex.

When reviewing previous studies on this topic, Lombardo et al.[19] examined the ratio of the lengths of the second and fourth toes in some male and female birds. In their study, they found no statistically significant differences in the ratios of the lengths of the second and fourth toes on the left foot of house sparrows, tree swallows, budgerigars, and chickens. Similarly, in Japanese quail, which also underwent analysis regarding this ratio, no statistically significant differences were found between the sexes. In the same study, no sexual dimorphism was observed in the ratios of the lengths of the second-third and third-fourth toes; however, it was noted that the second toe in male quail was more curved inward compared to females.[20] Additionally, Ruuskanen et al.[21] found that in their study of wild birds, the ratios of the lengths of the second-third and second-fourth toes were higher in males than in females. The differences in toe lengths between the sexes may be related to variations in hormone levels in males and females.[22,23] In studies comparing the right and left foot, a hypothesis was proposed suggesting that one leg may be used more than the other.[24] These findings contribute to a broader understanding of the factors influencing morphological variations among avian species and highlight the complexity of sexual dimorphism and its ecological implications.

Although there have been studies on birds concerning their skulls, beaks, and wings, there is a scarcity of geometric morphometric research focused specifically on their foot.

In the present study, we employed geometric morphometric methods to elucidate the phylogenetic relationships of the foot structures in partridges. A detailed shape analysis was conducted on the foot of chukar partridges and gray partridges, which are distinguished by their differing habitats. The obtained data indicate that the overall shape of partridge foot is significantly influenced by factors such as species, sex, and geographical region. Consequently, the findings from this study are expected to contribute to the classification of partridges, enhance our understanding of the adaptations they have undergone, and be utilized for insights into the identification of fauna and evolutionary processes. The low population density of chukar partridges in the study area is a limitation of this research. The scarcity of individuals may have affected the sample size, potentially limiting the statistical power and generalizability of the findings. Despite this limitation, the current results provide valuable insights into the morphological characteristics of the species and contribute to the existing literature.

As a result of, regardless of sex, the thumb length of chukar partridges was longer than that of grey partridges. When comparing species, the angle between the toes of female chukar partridges was wider than that of female grey partridges. Similarly, the toe angle of male grey partridges was wider than that of male chukar partridges. In terms of sex differences, the toe angle in male grey partridges was wider than in female grey partridges, while the toe angle in female chukar partridges exceeded that of male chukar partridges. The contribution of directional asymmetry to variation was lower than that of fluctuating asymmetry in both shape and size. This indicates that the observed asymmetry arises primarily from developmental differences rather than consistent lateralization.

Ethics Committee Approval: Ethical approval for the study was obtained from the Ethics Committee of Selçuk University Faculty of Veterinary Medicine (Date: 05/09/2024, Approval No: 2024/139).

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ATATÜRK ÜNİVERSİTESİ YAYINLARI ATATURK UNIVERSITY PUBLICATIONS

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A Bibliometric Analysis on Sustainability in Dairy Farming

Süt Sığırcılığında Sürdürülebilirlik Üzerine Bibliyometrik Bir Analiz

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ABSTRACT

Dairy farming is a crucial sector in terms of food security, economic development, and environmental sustainability. However, factors such as climate change, animal welfare, economic pressures, and environmental issues threaten the sustainability of the sector. This study aimed to analyze academic publications on sustainability in dairy farming using bibliometric analysis to determine research trends and gaps in the literature. In this study, 410 articles published between 1992 and 2025 in the Web of Science database were analyzed to identify scientific production trends, leading authors, journals, institutions, and countries in the field of dairy farming sustainability. The analysis results indicate a significant increase in the number of publications since 2017, with an annual growth rate of 6.07%, demonstrating the rising scientific output in this area. Keywords such as "sustainability," "dairy farming," and "economic sustainability" stand out, while Agricultural Systems and the Journal of Cleaner Production are among the leading journals in terms of citations and publications. The findings show that scientific production is geographically concentrated in countries such as the United States, Italy, and the Netherlands. The study reveals that knowledge gaps and regional disparities in the field may present potential research topics for future studies.

Keywords: Bibliometric analysis, dairy farming, economic sustainability, environmental impact, sustainability

ÖZ

Süt sığırcılığı, gıda güvenliği, ekonomik kalkınma ve çevresel sürdürülebilirlik açısından önemli bir sektördür. Ancak iklim değişikliği, hayvan refahı, ekonomik baskılar ve çevresel sorunlar gibi faktörler sektörün sürdürülebilirliğini tehdit etmektedir. Bu çalışmada, süt sığırcılığında sürdürülebilirlik konusundaki akademik yayınlar bibliyometrik analiz yöntemiyle incelenerek, araştırma eğilimleri ve literatürdeki boşlukların belirlenmesi amaçlanmaktadır. Bu çalışmada, 1992–2025 yılları arasında Web of Science veri tabanında yer alan 410 makale analiz edilerek, süt sığırcılığında sürdürülebilirlik konusundaki bilimsel üretim eğilimleri, önde gelen yazar, dergi, kurum ve ülkeler belirlenmiştir. Analiz sonuçları, 2017 yılından itibaren yayın sayısında belirgin bir artış yaşandığını ve yıllık %6,07'lik büyüme oranıyla bu alanda bilimsel üretimin arttığını göstermektedir. "sustainability", "dairy farming" ve "economic sustainability" gibi anahtar kelimeler öne çıkarken, Agricultural Systems ve Journal of Cleaner Production dergileri atıf ve yayın açısından önde gelen dergilerdir. Bulgular, bilimsel üretimin coğrafi olarak United States, Italy ve Netherlands gibi ülkelerde yoğunlaştığını göstermektedir. Çalışma, alandaki bilgi boşlukları ve bölgesel farkılıkların, gelecekteki araştırmalar için potansiyel çalışma konuları teşkil edebileceğini ortaya koymaktadır.

Anahtar Kelimeler: Bibliyometrik analiz, çevresel etki, ekonomik sürdürebilirlik, süt sığırcılığı

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INTRODUCTION

The livestock sector, which holds strategic importance for adequate and balanced nutrition, also has significant economic, social, and biological functions. It plays a critical role in ensuring the growing demand for food security and safety.[1-3] Milk and dairy products provide essential nutrients such as iron, sterols, and vitamins necessary for human growth and health, ranking among the world's primary food sources.[4-6]

Dairy farming is of great importance not only for food production but also for sustainable development. Its economic and sociological importance (such as rural employment, balanced urbanization, and migration) positions the sector as the driving force of animal production. However, global warming and climate change have brought the environmental impacts of dairy farming, particularly ruminant farming, into discussion.[7-10]

The pressures on business revenues, the emergence of animal diseases, concerns related to animal welfare, and environmental issues in the dairy farming sector have increased interest in the concept of sustainability.[11] Animal diseases raise treatment costs while negatively affecting milk yield and animal health. Additionally, dairy farming is closely associated with environmental factors such as greenhouse gas emissions, water consumption, and land use. The sustainable management of these factors plays a crucial role in reducing the sector's environmental impact.[12,13] Sustainable animal production plays a critical role in managing global challenges such as human population growth, food security, climate change, energy consumption, biodiversity, and the environmental impacts of human activities.[14,15]

In this context, the concept of sustainability in dairy farming is generally addressed through three fundamental dimensions: environmental, economic, and social sustainability.[16-18] Environmental sustainability encompasses factors such as greenhouse gas emissions, water consumption, land use, and biodiversity associated with dairy farming.[19,20] Economic sustainability includes dairy farms' profitability, financial resilience, and resistance to market fluctuations.[11,21,22] Social sustainability involves the well-being of individuals engaged in dairy farming, animal welfare and health, and rural development.[23,24]

In recent years, there has been a significant increase in academic studies on dairy farming and sustainability. Classifying and analyzing these studies within a systematic framework is crucial for identifying research trends in the field. In this regard, bibliometric analysis allows for the quantitative and qualitative examination of publications to evaluate the development of a specific academic field.[25,26]

Bibliometric analysis is a method utilized to examine academic studies on a particular topic based on factors, such as the number of publications, citation distribution, keywords, geographical distribution of research areas, and author collaborations. This analysis helps understand the evolution of scientific research over time, identify frequently studied topics, and detect gaps in the literature.[27]

This study aimed to conduct a bibliometric analysis of academic publications on sustainability in dairy farming to evaluate research trends, dominant themes, and geographical distributions.

MATERIALS AND METHODS

Data Source and Research Process

Ethics committee permissions for this study were obtained from Selçuk University Faculty of Veterinary Medicine, Experimental Animal Production and Research Centre Ethics Committee and the study was carried out within the scope of the permission of this committee dated 28/04/2025 and numbered 2025/52. The data source for this bibliometric analysis study was obtained from the Web of Science Core Collection (WoS; New York, USA). WoS serves as a standard tool for a significant portion of citation studies worldwide. Additionally, WoS publishes studies that comply with publication ethics.[28]

A literature search was conducted on January 8, 2025, using an advanced search strategy. A total of 728 articles in agricultural and veterinary sciences were retrieved using the keywords "sustainability" AND "dairy farm" OR "dairy farming" OR "dairy cattle." The inclusion criteria were "document type: article" and "language: English," which resulted in 570 articles. Upon reviewing the content of the retrieved articles, 160 studies were excluded due to irrelevance to dairy farming sustainability, word similarities, or research on different animal species. After applying all filters, the bibliographic dataset consisted of 410 articles fulfilling the required criteria (Figure 1).

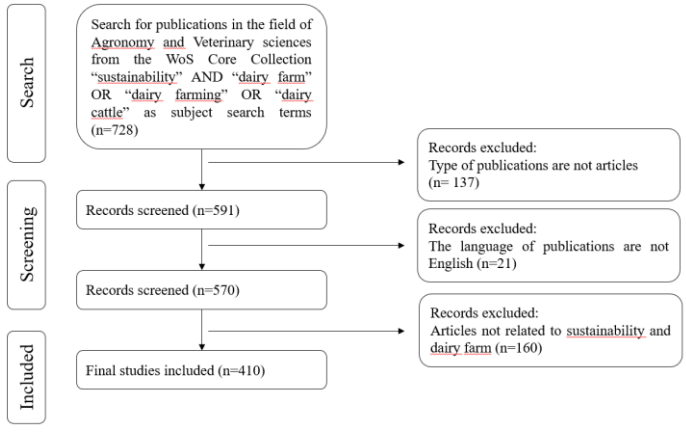

Figure 1. Workflow chart.

The data for this bibliometric analysis were extracted from the WoS using the "Full record and cited references" option in both "plain text" and "bib text" formats. The bibliometric analysis of the data was conducted using the "bibliometrics" package available in the R version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria) statistical program, along with the Biblioshiny interface and Excel 2022. Biblioshiny is a tool that offers multiple categorization options based on sources, authors, documents, social structure, conceptual structure, and intellectual structure.[29]

Bibliometric Methodology

Bibliometric analysis is a tool used to measure the scientific output of various academic elements (studies, authors, journals, keywords, institutions, and countries) in a particular field and to visually present their intellectual, social, and conceptual structures.[30] Bibliometric analysis consists of two main stages: performance analysis and scientific mapping.[31] Performance analysis examines the contributions of scientific elements within a field using specific metrics (such as citation count, publication count, and h-index).[32] Scientific mapping, on the other hand, visually presents the intellectual and structural networks among scientific elements.[33]

RESULTS

General descriptive information about the data obtained from WoS and document types is presented in Table 1. Between 1992 and 2025, a total of 410 studies from 144 different journals were included in the analysis. The total number of authors was 1,539, with 20 single-authored studies and 390 multi-authored studies. The studies in the dataset contained a total of 17,494 references, with an average citation count of 16.11 per article. The bibliographic data included 1,033 keywords plus and 1,295 author keywords. An examination of author collaboration statistics revealed an average of 4.38 co-authors per article, an international co-authorship rate of 32.20%, and a collaboration index of 3.89.

Table 1. General Descriptive Information

Description	Results
Main Information About Data	
Timespan	1992:2025
Sources (Journals, Books, etc.)	144
Documents	410
Annual growth rate (%)	6.07
Document Average Age	7.65
Average citations per documents	16.11
References	17494
DOCUMENT TYPES	
Article	383
Article; early access	4
Article; proceedings paper	23
Document Contents	
Keywords Plus (ID)	1033
Author's Keywords (DE)	1295
Authors	1539
Authors appearances	1796
Authors of single-authored documents	20
Authors of multi-authored documents	1519
Authors Collaboration	
Single-authored documents	20
Multi-authored documents	390
Authors per document	3.75
Documents per author	0.26
Co-Authors per documents	4.38
Collaboration index	3.89
Author footprint index	0.15
International co-authorships (%)	32.20

The publication trends of studies on sustainability in dairy farming between 1992 and 2025 show a significant increase in the number of articles over the years. The first article on this topic was published in 1992. In 2024, 62 articles were published, marking the highest annual count. The annual growth rate was 6.07%. However, the growth trend was not linear. Until 2016, the average annual number of publications was 5.16, while a sharp increase occurred from 2017 onwards, with a continuing upward trend in publication numbers (Figure 2).

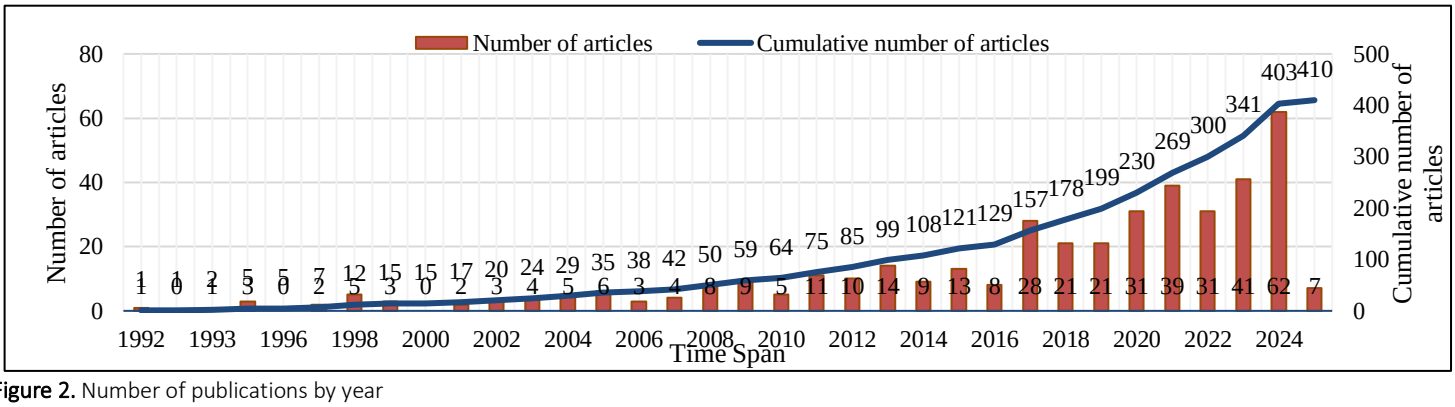

Figure 2. Number of publications by year

A three-field diagram was used to provide a general assessment of publications on sustainability in dairy farming between 1992 and 2025. This diagram visualizes the relationships among three main elements: sources (SO), authors (AU), and keywords (DE). Authors predominantly used keywords such as sustainability, dairy farming, dairy farm, milk, and environment. The most preferred journal was Agricultural Systems, while leading authors such as Tamburini A. and Sandrucci A. primarily published their studies in the Italian Journal of Animal Science (Figure 3).

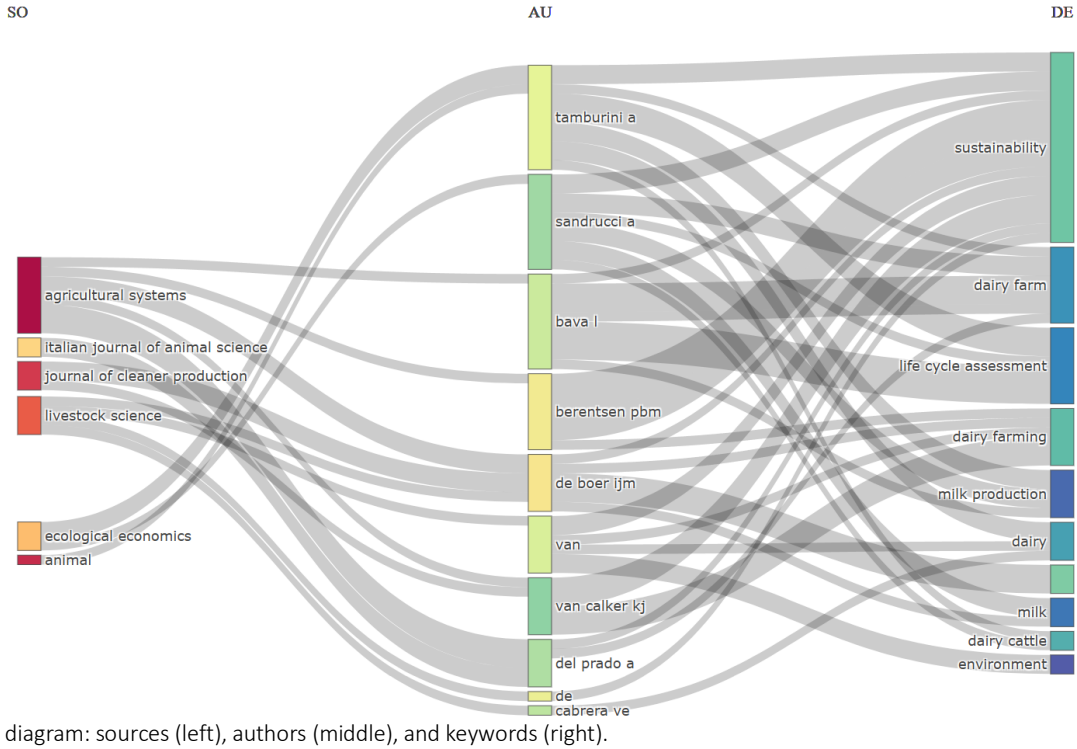

Figure 3. Three-field diagram: sources (left), authors (middle), and keywords (right).

According to the findings, articles on sustainability in dairy farming were published in 144 different journals. The top 10 journals in which these articles were published are listed in Table 2. Agricultural Systems (n = 31) and Journal of Cleaner Production (n = 19) were the most productive journals, while Journal of Dairy Science (n = 663) and Animal (n = 563) were the most highly cited journals. The top 10 journals ranked by h-index accounted for approximately 43% of all publications in this field. The international collaboration rates for these journals exceeded 30%.

Table 2. Top 10 Journals Contributing to Publications on Sustainability in Dairy Farming

Source	h_index	g_index	m_index	TC	NP	CI	IC (%)	JIF	JIF Quartile	Country
Agricultural Systems	16	24	0.727	611	31	19.71	53.55	6.1	Q1	Switzerland
Journal of Cleaner Production	13	19	0.813	522	19	27.47	33.02	9.8	Q1	USA
Journal of Dairy Science	13	25	0.419	663	26	25.5	31.96	3.7	Q1	USA
Animal	12	20	0.667	563	20	28.15	39.44	4	Q1	England
Sustainability	12	17	1	383	40	9.575	30.63	3.3	Q2	Switzerland
Animals	6	11	0.667	124	16	7.75	31.40	2.7	Q1	Switzerland
Agronomy for Sustainable Development	5	6	0.278	241	6	40.17	50.79	6.4	Q1	France
Ecological Economics	5	5	0.156	305	5	61	41.10	6.6	Q1	Netherlands
International Journal of Agricultural Sustainability	5	6	0.333	88	6	14.67	54.83	3.3	Q1	England
Livestock Science	5	7	0.333	125	7	17.86	34.21	1.8	Q2	Netherlands

NP = number of publications, TC = total citations, CI = citation impact, IC = international collaborations, JIF = journal impact factor.

The metrics demonstrating the scientific productivity of the top 10 authors in publications on sustainability in dairy farming are provided in Table 3. The h-index of these authors ranged from 3 to 6. De Boer, I.J.M. was the most productive author (n = 7), while Berentsen, P.B.M. had the highest citation count (n = 350). Additionally, Baes, C.F. had the highest citation impact. Among the top 10 authors in this field, four were affiliated with institutions in the Netherlands. According to Lotka's Law, 60% of authors were expected to contribute with one publication, 15% with two publications, and 7% with three publications.[34] However, in this field, 87.4% of authors contributed with one publication, 9.7% with two publications, and 2.1% with three publications, indicating a deviation from Lotka's Law. This discrepancy can be attributed to the fact that 51% of the studies in this field were published within the last five years. Additionally, based on Lotka's Law, authors with more than four publications in this field could be considered core contributors.

Table 3. Top 10 Most Productive Authors

Author	h_index	g_index	m_index	TC	NP	CI	Country
De Boer, I.J.M.	6	7	0.333	174	7	24.86	Netherlands
Berentsen, P.B.M.	5	5	0.227	350	5	70.00	Netherlands
Sandrucci, A.	4	4	0.235	128	4	32.00	Italy
Tamburini, A.	4	5	0.235	128	5	25.60	Italy
Van Calker, K.J.	4	4	0.182	314	4	78.50	Netherlands
Van Keulen, H.	4	4	0.148	59	4	14.75	Netherlands
Baes, C.F.	3	3	0.333	338	3	112.67	Switzerland
Bava, L.	3	4	0.231	192	4	48.00	Italy
Cabrera, V.E.	3	4	0.143	54	4	13.50	USA
Casey J.	3	3	0.250	67	3	22.33	Belgium

NP= number of publications, TC= total citations, CI= citation impact.

The top 10 most productive institutions, ranked by the number of publications on sustainability in dairy farming, are listed in Table 4. A total of 592 institutions were identified among the authors, indicating a high level of collaboration among researchers from different institutions. The top two institutions with the highest number of publications were Wageningen University (Netherlands) and the University of Milan (Italy).

Table 4. Top 10 Most Productive Institutions

Affiliation	Articles	Country
Wageningen University	31	Netherlands
University of Milan	29	Italy
Wageningen University and Research Center	28	Netherlands
Wageningen University and Research	23	Netherlands
University of Guelph	22	Canada
The Swedish University of Agricultural Sciences	18	Sweden
Animal and Grassland Research and Innovation Center	17	Ireland
University of Wisconsin	17	USA
University of Toulouse	16	France
University College Dublin	15	Ireland

From 1992 to 2025, a total of 56 countries contributed to publications on dairy farming sustainability. A heatmap visualized the geographical distribution of publications by country (Figure 3A). The top three countries with the highest number of publications were the United States (n = 45), Italy (n = 44), and the Netherlands (n = 30). Other productive countries included Canada, Brazil, China, and several European countries (Ireland, France, the United

Kingdom, and Spain). Africa, Central and Western Asia, the Caribbean, and Eastern European countries were significantly underrepresented. An analysis of the authors' countries showed that the United States, Italy, and the Netherlands accounted for 29% of the publications in this field. The multiple-country publication (MCP) percentage was used to reflect international collaboration. In leading countries, including the United States, Italy, and the Netherlands, most publications were single-country publications (SCP). Among the top ten most productive countries, only the United Kingdom had an MCP rate exceeding 50% (53.3%) (Figure 3B). Regarding citations, the top five most cited countries were the Netherlands (n = 914), Italy (n = 767), the United States (n = 741), Canada (n = 628), and Belgium (n = 515). In terms of average citations per document, the top five countries were Canada (48.30), Kenya (47.50), Belgium (46.80), the Netherlands (30.50), and New Zealand (23.50) (Figure 3C).

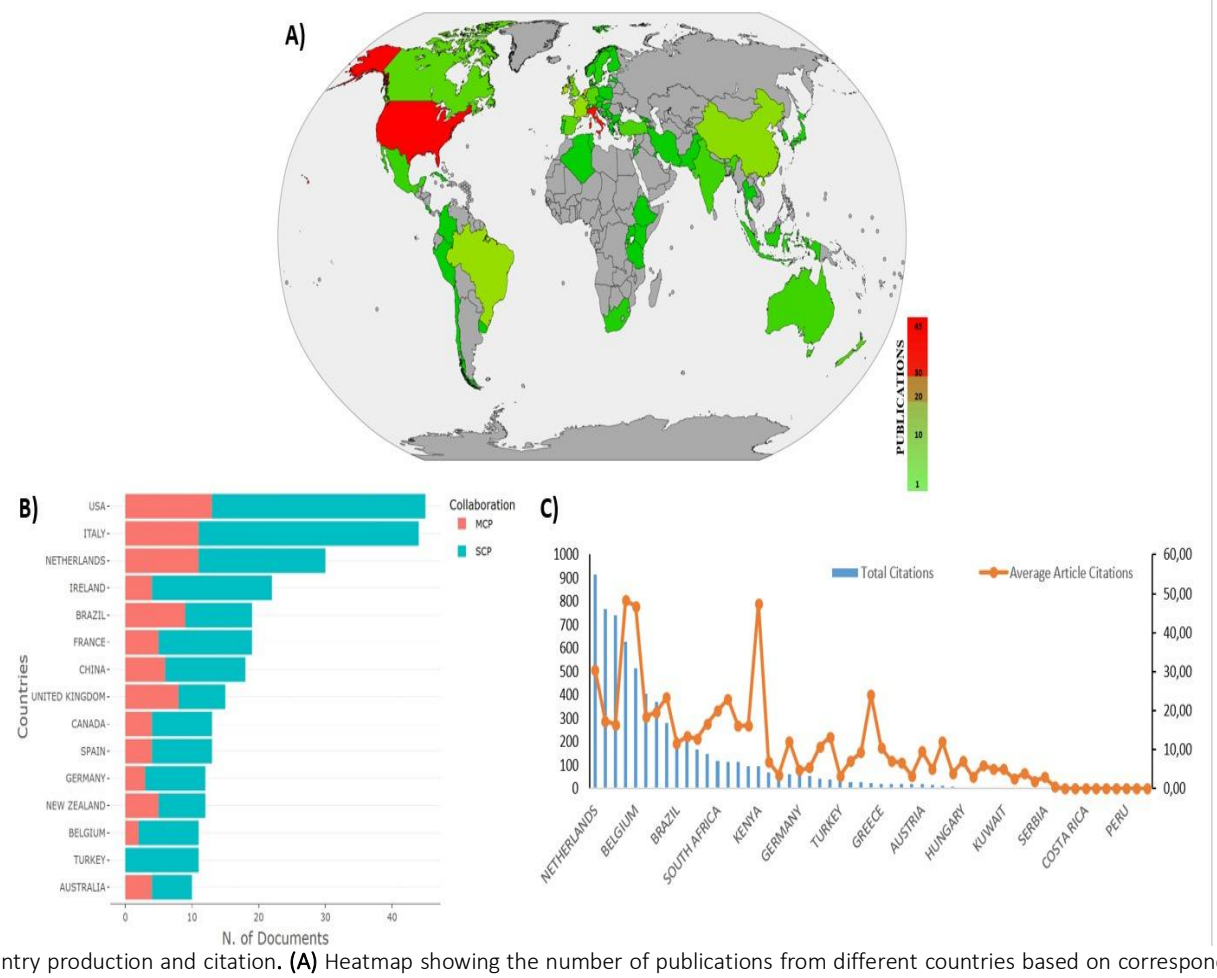

Figure 4. Country production and citation. **(A)** Heatmap showing the number of publications from different countries based on corresponding authors' affiliations. **(B)** Countries of corresponding authors, SCP (single-country publication); MCP (multiple-country publication). **(C)** Most cited countries.

Keyword analysis is essential for identifying research trends in a field. The bibliometric network of the most frequently used keywords is presented in Figure 5. Similar keywords were clustered based on network analysis, forming seven main clusters represented in red, green, yellow, purple, blue, pink, and brown. The keyword network analysis revealed that the terms “sustainability,” “economic sustainability,” “sustainable agriculture,” “dairy farming,” and “dairy” were frequently used.

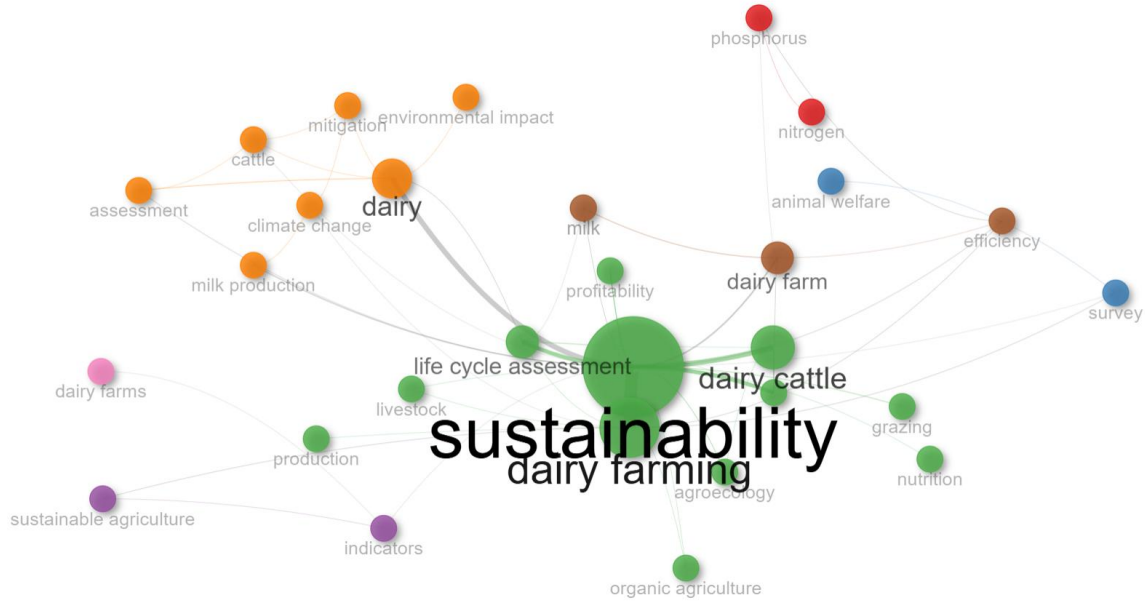

Figure 5. Bibliometric network of the most frequently used keywords.

Table 5 lists the ten most cited references. The study titled "A 100-Year Review: Identification and Genetic Selection of Economically Important Traits in Dairy Cattle" by Miglior F., published in 2017, ranked first with 292 citations, leading both in total citations and annual average citations. The study "MOTIFS: a monitoring tool for integrated farm sustainability" by Meul M., published in 2008, ranked second with 159 citations.

Table 5. Top ten most cited references

Paper	DOI	TC	TC per Year
Miglior F., 2017, J. Dairy Sci.	10.3168/jds.2017-12968	292	32.44
Meul M., 2008, Agron. Sustain. Dev.	10.1051/agro:2008001	159	8.83
Van Passel S., 2007, Ecol. Econ.	10.1016/j.ecolecon.2006.06.008	144	7.58
De Vries A., 2020, Animal	10.1017/S1751731119003264	130	21.67
Sanderson M.A., 2005, Agron. J.	10.2134/agronj2005.0032	126	6.00
Van Calker K.J., 2005, Agric. Human Values	10.1004/s10460-004-7230-3	118	5.62
Guerci M., 2013, J. Clean. Prod.	10.1016/j.jclepro.2013.04.035	104	8.00
Boichard D., 2012, Animal	10.1017/S1751731112000018	96	6.86
Schuppli C.A., 2014, J. Anim. Sci.	10.2527/jas.2014-7725	92	7.67
Van Calker K.J., 2004, Agric. Syst.	10.1016/j.agsy.2004.02.001	90	4.09

TC= total citations

DISCUSSION

The analysis results not only demonstrate the increasing scientific productivity in the field of dairy farming sustainability but also highlight significant geographical disparities in research distribution. Notably, a sharp increase in publication numbers after 2017 stands out. The annual growth rate in this field was determined to be 6.07%. In recent years, growing awareness of the environmental challenges posed by agricultural production, ongoing economic competition affecting farm incomes, and concerns regarding animal welfare have contributed to the rising interest in dairy farming sustainability.[11] The implementation of the United Nations Sustainable Development Goals (SDGs) in 2015 has also played a role in increasing attention to this subject.[35]

An analysis of the literature distribution reveals that the

variation among the top ten contributing journals indicates the multidisciplinary nature of research on dairy farming sustainability. The distribution of authors and collaboration statistics further demonstrate the openness of the field to cooperation. The predominance of multi-author studies over single-author ones and an international collaboration rate of 32.20% suggest a high tendency among researchers from different institutions and regions to share knowledge and expertise. This collaborative approach is a key factor in enhancing scientific productivity and addressing challenges in sustainable dairy farming. The presence of a critical global issue such as climate change encourages researchers to develop joint solutions and promotes interdisciplinary studies.

At the country level, the fact that the majority of publications originate from countries such as the United States, Italy, and the Netherlands indicates that developed countries have a strong interest in and high research capacity for this topic. In the United States, advancements in dairy farm technology and management over the past 50 years have led to increased milk production while reducing environmental impacts.[36] Italy's significant role in European dairy production and the European Union's policies promoting sustainable production have contributed to the concentration of research in this area.[37] In the Netherlands, the institutionalization of cooperatives, the implementation of the EU's Common Agricultural Policy in the 1960s, regulatory measures, and government support for dairy farming have accelerated sustainability-related research.[38] Although two of the top three contributing countries are in Europe, economic sustainability challenges vary across European nations. Countries such as Romania, Lithuania, Croatia, Austria, Poland, and Bulgaria, where small-scale farms are prevalent, have been reported to lag in this area.[39] In developing countries, the dairy farming sector faces various structural and economic challenges that threaten sustainability, including financial constraints, insufficient private sector investments, lack of technology and research, and inadequate cold chain and marketing infrastructure.[40-42]

The United States, Italy, and the Netherlands demonstrate a high level of international collaboration in sustainability studies related to dairy farming. In contrast, studies in Turkiye are mostly conducted at the national level. Citation counts indicate the scientific impact of a country's publications. To enhance the citation impact of sustainability studies in dairy farming in countries like Turkiye, greater international collaboration and publication in high-impact journals are necessary. Studies conducted through international collaborations tend to attract more global attention and receive higher citation counts.[43,44] Notably, despite a lower number of publications, Kenya stands out with a high citation count. The high average citation rate of studies from Kenya suggests that research conducted in this country is particularly significant for local and regional sustainability practices. Furthermore, the increasing focus on this topic among academic researchers is likely influenced by the tangible effects of climate change in the region.[45] This finding underscores that a small number of studies can be highly impactful in terms of quality and influence, highlighting the importance of assessing research outputs not only quantitatively but also qualitatively.

Bibliometric network analysis of keywords is crucial for understanding research trends and the conceptual structure of the field. The frequent use of terms such as "sustainability," "economic sustainability," "dairy farming," and "dairy" reflects the core research focus areas. Keyword clusters indicate that researchers approach the topic from different perspectives, such as economic and environmental aspects, pointing to potential research directions for future studies. Douglas et al.[35] reported that the term "dairy farming" appears in multiple keyword clusters, emphasizing its central role in the field due to the diverse aspects examined by researchers. Similarly, in this study, the terms "dairy farming" and "dairy cattle" were found in different keyword clusters, indicating that research in the field encompasses the environmental, social, and economic dimensions of dairy farming. Keywords such as "methane," "climate change," "carbon," and "footprint" suggest that the impact of dairy farming on greenhouse gas emissions and climate is a frequent research subject. The term "animal welfare" is also considered a significant component of the social dimension of sustainability in dairy farming. While the dairy sector faces challenges in maintaining a high quality of life, minimizing environmental impact, and ensuring cost-effective production, global climate agreements and consumer expectations regarding dairy production continue to shape research in this field.[13] A review of the literature reveals that studies integrating all three dimensions of sustainability—environmental, social, and economic—are limited.[17] Life cycle assessment is an internationally recognized method used to comprehensively evaluate the environmental impact of sustainability.[46] The co-occurrence of the keywords "sustainability," "life cycle assessment," and "dairy cattle" indicates that life cycle assessment is widely utilized in sustainability assessments of dairy farming.

In conclusion, the data obtained indicate that the literature on sustainability in dairy farming is rapidly evolving both quantitatively and qualitatively, with the conceptual and intellectual structure of the field transforming over time. The study's results also highlight knowledge gaps and

regional disparities, suggesting potential research areas for future studies. Moreover, bibliometric analysis provides a comprehensive assessment of scientific productivity and collaboration networks, serving as a valuable tool for strategic planning in the development of sustainable dairy farming practices and policies. Researchers interested in this field can enhance their contributions by collaborating with leading countries, regions, and authors and carefully selecting target journals for publication.

The methodological approach of this study, supported by the reliability of the WoS database and advanced bibliometric analysis tools, comprehensively reveals the scientific productivity, collaboration networks, and intellectual structure of the field. However, the study has certain limitations. The inclusion of only English-language publications may have excluded some regional literature from the analysis. Additionally, as the coverage of the WoS database does not encompass all studies available in other databases, the generalizability of the results may be subject to certain constraints.

Ethics Committee Approval: Ethics committee permissions for this study were obtained from Selçuk University Faculty of Veterinary Medicine, Experimental Animal Production and Research Centre Ethics Committee and the study was carried out within the scope of the permission of this committee dated 28/04/2025 and numbered 2025/52.

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Author Contributions: Concept - AG; Design - BNC; Supervision - AG; Resources - BNC; Materials - BNC; Data Collection and/or Processing - HS; Analysis and/or Interpretation - HS; Literature Search - HS; Writing Manuscript - BNC; Critical Review - AG; Other – BNC

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Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir - AG; Tasarım - BNC; Denetleme - AG; Kaynaklar - BNC; Materials - BNC; Veri Toplanması ve/veya İşlemesi - HS; Analiz ve/ veya Yorum - HS; Literatür Taraması - HS; Yazıyı Yazan - BNC; Eleştirel İnceleme - AG; Other – BNC

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Protective Potential of Morin in Ifosfamide-Induced Lung Toxicity: Modulation of Oxidative Stress, Inflammation and Apoptosis Parameters

İfosfamid ile İndüklenen Akciğer Toksisitesinde Morin'in Koruyucu Potansiyeli: Oksidatif Stres, İnflamasyon ve Apoptoz Parametrelerinin Modülasyonu

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ABSTRACT

In this study, the protective effect of morin against lung toxicity induced by ifosfamide (IFO), a widely used drug in cancer treatment, was investigated. A total of thirty-five male Sprague-Dawley rats were randomly distributed into five experimental groups: Control, Morin (200 mg/kg), IFO and two different morin doses (IFO + Morin 100 mg/kg and IFO + Morin 200 mg/kg). Rats were given morin 100 mg/kg or 200 mg/kg for 2 days and on the second day, IFO 500 mg/kg was administered as a single dose. Markers of oxidative stress, inflammation, autophagy and apoptosis were analyzed using biochemical methods. According to the data obtained, IFO increased malondialdehyde (MDA) levels in lung tissue, while decreasing superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) activities and glutathione (GSH) levels. However, it was observed that morin administration decreased MDA levels and increased GSH levels and GPx, SOD, CAT activities. IFO administration inhibited the expression of B-cell lymphoma-2 (Bcl-2) and the nuclear factor erythroid 2-related factor 2 (Nrf-2) /heme oxygenase-1 (HO-1) pathway, while increasing levels of nuclear factor-kappa B (NF-κB), inducible nitric oxide synthase (iNOS), Bcl-2-associated x protein (Bax), and cysteine aspartate specific protease-3 (Caspase-3). However, morin decreased NF-κB and iNOS levels and inhibited inflammation by activating the Nrf-2/HO-1 pathway and prevented apoptosis by decreasing Bax and Caspase-3 and increasing Bcl-2. It also caused a decrease in Beclin-1 levels. According to the findings, IFO by affecting various signaling pathways in lung tissue, causing cellular damage, while morin demonstrated protective qualities against this damage.

Keywords: Apoptosis, ifosfamide, inflammation, lung toxicity, morin, oxidative stress

ÖZ

Bu çalışmada, kanser tedavisinde yaygın olarak kullanılan bir ilaç olan İfosfamid (IFO)'in neden olduğu akciğer toksisitesine karşı morin'in koruyucu etkisi araştırıldı. Toplam otuz beş erkek Sprague-Dawley sıçanı rastgele olarak beş deney grubuna ayrıldı: Kontrol, Morin (200 mg/kg), IFO ve iki farklı Morin dozu (IFO + Morin 100 mg/kg ve IFO + Morin 200 mg/kg) uygulandı. Sıçanlara 2 gün boyunca Morin 100 mg/kg veya 200 mg/kg verildi ve ikinci gün IFO 500 mg/kg tek doz olarak uygulandı. Oksidatif stres, inflamasyon, otofaji ve apoptoz belirteçleri biyokimyasal yöntemlerle analiz edildi. Elde edilen verilere göre, IFO akciğer dokusunda malondialdehit (MDA) seviyelerini artırırken, süperoksit dismutaz (SOD), katalaz (KAT), glutatyon peroksidaz (GPx) aktiviteleri ve glutatyon (GSH) seviyelerini azalttı. Ancak morin uygulamasının MDA düzeyini azalttığı, GSH seviyeleri ve GPx, SOD, KAT aktivitelerini arttırdığı gözlemlendi. IFO uygulaması, B-hücreli lenfoma-2 (Bcl-2) ve nükleer faktör eritroid 2 ile ilişkili faktör 2 (Nrf-2) /hem oksijenaz-1 (HO-1) yolunun ekspresyonunu inhibe ederken, nükleer faktör-kappa B (NF-κB), indüklenebilir nitrik oksit sentaz (iNOS), Bcl-2 ile ilişkili x proteini (Bax) ve sistein aspartat spesifik proteaz-3 (Kaspaz-3) seviyelerini artırdı. Ancak morin NF-κB ve iNOS seviyelerini azalttı ve Nrf-2/HO-1 yolağını aktive ederek inflamasyonu inhibe etti ve Bax ve Kaspaz-3'ü azaltıp Bcl-2'yi artırarak apoptozu engelledi. Ayrıca Beklin-1 düzeylerinde azalmaya neden oldu. Bulgulara göre, IFO akciğer dokusunda çeşitli sinyal yollarını etkileyerek hücresel hasara neden olurken, morin bu hasara karşı koruyucu özellik gösterdi.

Anahtar Kelimeler: Akciğer toksisitesi, apoptoz, ifosfamid, inflamasyon, morin, oksidatif stres

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INTRODUCTION

Cytostatics and anti-neoplastics are anticancer medications with potent modes of action.[1] The medication works by preventing DNA synthesis or interfering with cell division. Apoptosis results from cell damage that cannot be repaired. However, the primary goal is for the medication to predominantly target cancer cells.[2] Ifosfamide (IFO), an alkylating drug from the oxazaphosphorine class, is used in conjunction with other anticancer medications to treat a range of solid tumors, such as lymphoma, small cell lung cancer, testicular cancer, soft tissue sarcoma, osteosarcoma, bladder cancer, cervical cancer, and ovarian cancer.[3] Like other cytostatic cancer drugs, IFO has serious and life-threatening side effects that limit its therapeutic value.[4] Myelosuppression, interstitial pneumonia, hemorrhagic cystitis, alopecia, vomiting, nausea, and arrhythmia are among the common adverse effects of IFO.[5] The hazardous metabolites 4-hydroxycyclophosphamide, acrolein, and phosphoramide mustard are produced in the liver and, to a lesser extent, in the lung from IFO, an analog of cyclophosphamide.[6] Mechanisms of this toxicity may include disruptions in pro-oxidant/antioxidant balance, enhancement of inflammatory pathways and modulation of apoptotic signaling in pulmonary tissues.[7]

Due to the harmful consequences of chemotherapy medications used in cancer treatment, efforts to discover novel cancer drugs have surged recently.[1] Since ancient times, medicinal plants have been used as therapeutic agents in the treatment of various human diseases due to their antibacterial, antioxidant, anti-inflammatory, antiapoptotic and antiproliferative effects.[8] Flavanoids are polyphenolic compounds found in many fruits, vegetables and plant roots.[9] White mulberry, fig, and cranberry branches are rich in morin, a flavonoid molecule that works by lessening the harmful effects of anticancer medications on cancer cells.[10] Morin has several pharmacological properties such as reducing oxidative stress by scavenging free radicals, preventing apoptosis by reducing the release of apoptotic factors from mitochondria.[11] When these beneficial pharmacologic effects of morin are evaluated, it can be considered that morin can be used for protective administrationtion in lung injury after IFO use. Nevertheless, there is no research on morin's protective properties against in the lung IFO damage.

In this study, biochemical parameters such as oxidative stress, inflammation, DNA damage, and apoptosis were analyzed to evaluate the potential protective effects of morin against IFO-induced pulmonary injury in male rats.

MATERIALS AND METHODS

Chemicals

IFO (Holoxan) was obtained through Eczacıbaşı, Istanbul, Turkey. Morin and other chemical reagents were obtained from Sigma-Aldrich, St. Louis, Missouri, USA.

Animals

Thirty-five male Sprague Dawley rats, weighing 200-250 g, were utilized in this investigation. They were acquired from the Erzurum Atatürk University Experimental Animals Center. Rats were housed in a room temperature of 24-25°C and a humidity of 45 ± 5%. The rats were given to adapt to the environment for one week before starting the experiment and were given as much water and standard food as they wanted throughout the experiment.

Experimental Design

Rats were split into five groups at random, each with seven rats:

Group I (Control): For two days, the rats were given oral saline; as a control, they were given a single dose of normal saline (1 mL i.p) on day two.

Group II (Morin): Rats were orally administered morin (200 mg/kg/day) for two days.[5]

Group III (IFO): Rats were given oral saline for two days, followed by a single dosage of IFO (500 mg/kg, i.p.) on day two to cause lung damage.[12]

Group IV (IFO+Morin 100): Rats were administered morin (100 mg/kg/day; p.o.) orally for two days and given a single dose of IFO (500 mg/kg) intraperitoneally on day two.

Group V (IFO+Morin 200): Rats were administered morin (200 mg/kg/day; p.o.) orally for two days and given a single dose of IFO (500 mg/kg, i.p.) intraperitoneally on day two.

24 hours following the final dose, the rats were killed under sevoflurane anesthesia was administered. The obtained lung tissues were preserved at -80°C for biochemical analyses.

Lipid Peroxidation and Antioxidant Activity Measurements

Lung tissue homogenates, needed to evaluate oxidative stress markers, were prepared according to the methodology indicated in our previous study.[13] Supernatants of lung tissue homogenates were analyzed for glutathione (GSH) levels by the Sedlak & Lindsay method,[14] for catalase (CAT) activity by the Aebi method,[15] for superoxide dismutase (SOD) activity by the Sun et al. method,[16] for glutathione peroxidase (GP_X) activity by the Lawrence and Burk method,[17] for malondialdehyde (MDA) content by the Placer et al. method,[18] and for total protein analysis by the Lowry et al. method.[19]

Determination of ELISA Markers

Nuclear factor-kappa B (NF-κB) (Cat. No: 201-11-0288), nuclear factor erythroid 2-related factor 2 (Nrf2) (Cat. No: 201-11-5375), heme oxygenase-1 (HO-1) (Cat. No: 201-11-0677), cysteine aspartate specific protease-3 (Caspase-3) (Cat. No: 201-11-5114), B-cell lymphoma-2 (Bcl-2) (Cat. No: 201-11-0038), Bcl-2-associated x protein (Bax) (Cat. No: 201-11-0035), inducible nitric oxide synthase (iNOS) (Cat. No: 201-11-0741), and Beclin-1 (Cat. No: 201-11-1689) (Sunred Biological Technology, Shanghai, China) were measured in lung tissue using rat ELISA kits. A measurement of absorbance was made at 450 nm.

Statistical Analysis

The Tukey test was used to identify group differences, while the one-way analysis of variance (ANOVA) test was employed to establish statistical differences and significant levels. All values are given as mean ± standard error (±SEM), while results at (P < .05) were considered significant. SPSS 20.0 (IBM SPSS Corp., Armonk, NY, USA) package program was used for these statistical analyses.

RESULTS

The effects of morin against the oxidative damage caused by IFO in the lung tissue were evaluated by oxidant marker MDA, antioxidant enzymes SOD, CAT, GPx activities and non-enzymatic marker GSH analysis and the findings are presented in Table 1. When we examined oxidative stress and lipid peroxidation in IFO-induced lung injury, we found that IFO significantly increased MDA levels and decreased SOD, CAT, GPx and GSH levels compared to control and morin groups (P < .05). On the other hand, IFO+Morin100 and IFO+Morin200 administration combined with IFO was found to decrease MDA levels while significantly increasing SOD, CAT and GPx activities and GSH levels (P < .05). It was determined that IFO+Morin200 was more effective in increasing CAT, GSH, and GPx levels compared to IFO+Morin100 (P < .05). However, there was no significant difference in SOD levels between the IFO+Morin100 group and the IFO+Morin200 group (P > .05).

Table 1. Effect of IFO and Morin on lung tissue MDA and GSH levels, SOD, CAT and GPx enzyme activities in each group.

Parameters	Control	Morin	IFO	IFO+Morin100	IFO+Morin200
MDA (nmol/g tissue)	22.20±0.48^{a}	23.14±0.55^{ab}	35.19±0.66^{d}	29.15±0.54^{c}	24.93±0.38^{ab}
GSH (nmol/g tissue)	1.81±0.04^{d}	1.85±0.02^{d}	0.77±0.02^{a}	1.04±0.03^{b}	1.45±0.02^{c}
SOD (U/g protein)	18.83±0.30^{c}	19.29±0.45^{c}	9.11±0.26^{a}	13.80±0.28^{b}	15.17±0.44^{b}
CAT (catal/g protein)	32.50±0.63^{d}	32.78±0.69^{d}	19.64±0.38^{a}	23.43±0.37^{b}	28.70±0.35^{c}
GPx (U/g protein)	26.83±0.62^{d}	27.59±0.58^{d}	15.82±0.32^{a}	19.64±0.25^{b}	24.11±0.44^{c}

Each group's values are given as the mean ± S.E.M. of seven rats. Significant differences between each group are indicated by different superscripts (a–d) in the same row (P < .05). MDA: malondialdehyde; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; IFO: ifosfamide.

Evaluation of Inflammation Markers

To investigate the effect of morin administration on IFO-triggered inflammatory response, NF-κB, Nrf-2, HO-1 and iNOS levels in lung tissue were evaluated and presented in Figure 1. Compared to the control and morin groups, NF-κB (Figure 1a), Nrf-2 (Figure 1b) and iNOS (Figure 1d) levels increased, while HO-1 (Figure 1c) levels decreased in the IFO group (P < .05). These levels were found to decrease at doses of 100 and 200 mg/kg morin administered together with IFO (P < .05).

Evaluation of Apoptosis Markers

To investigate the biomolecular mechanisms of the anti-apoptotic effects of morin on IFO-induced apoptosis in lung tissue, protein levels of proapoptotic Bax and antiapoptotic Bcl-2 levels, and Caspase-3 were investigated and presented in Figure 2. According to the data, IFO down-regulated Bcl-2 (Figure 2b), while increasing Bax (Figure 2a) and Caspase-3 (Figure 2c) levels (P < .05). Following morin administration, it was found that Bcl-2 was elevated to prevent IFO-induced apoptosis and that Bax and Caspase-3 were inhibited. Furthermore, the findings suggest that high doses of morin provide a more pronounced protective effect on apoptosis (P < .05).

Evaluation of Autophagy Marker

When beclin-1 levels, the most important autophagy marker, were examined, it was found that Beclin-1 levels increased in the lung tissue in the IFO-administration group (P < .05) and 100 and 200 doses of morin administered together with IFO decreased Beclin-1 levels and suppressed autophagy (P < .05). Beclin-1 levels results are given in Figure 3.

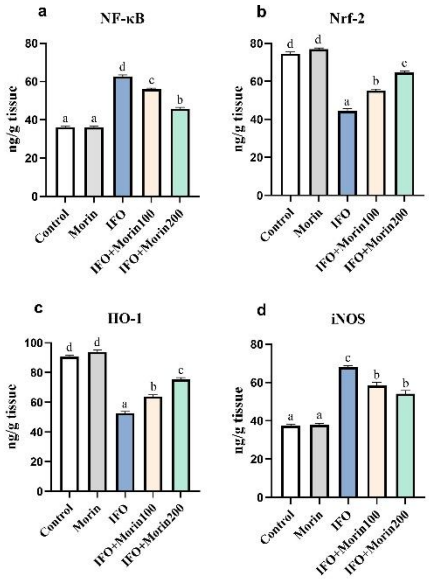

Figure 1. Lung tissue NF-κB (a), Nrf-2 (b), HO-1 (c), iNOS (d) levels after IFO and Morin administrations to rats. Each group's values are given as the mean ± S.E.M. of seven rats. Significant differences between each group are indicated by different superscripts (a–d) in the same row ($P < .05$).

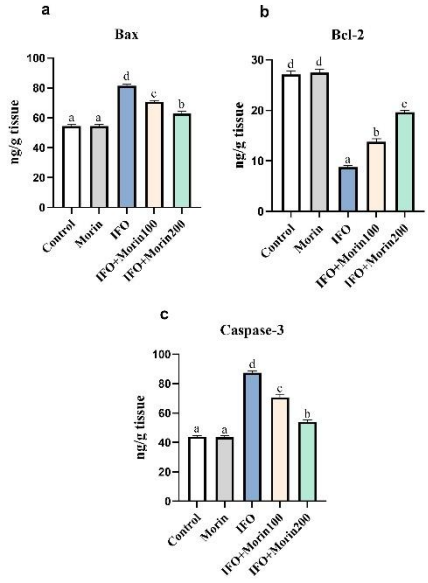

Figure 2. Lung tissue Bax (a), Bcl-2 (b) and Caspase-3 (c) levels after IFO and Morin administrations to rats. Each group's values are given as the mean ± S.E.M. of seven rats. Significant differences between each group are indicated by different superscripts (a–d) in the same row ($P < .05$).

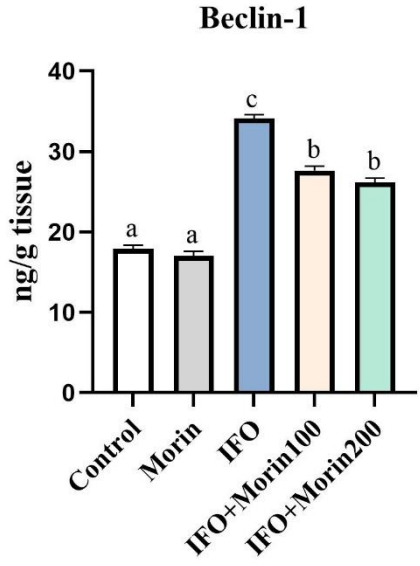

Figure 3. Lung tissue Beclin-1 levels after IFO and Morin administrations to rats. Each group's values are given as the mean ± S.E.M. of seven rats. Significant differences between each group are indicated by different superscripts (a–d) in the same row ($P < .05$).

DISCUSSION

With the use of antineoplastic agents in cancer treatment, the possible toxicity of these agents has also started to be evaluated.[20] Most antineoplastic agents have the potential to cause pulmonary toxicity by primarily affecting the lung parenchyma. In contrast, the airways, pleura and pulmonary circulatory system are more rarely affected.[21]

IFO, a cyclophosphamide analog, is commonly used to treat non-small cell lung cancer.[22] Although there have been several studies on the toxicity of IFO,[23, 24] lung injury has not been adequately described. Morin, an important flavonoid, has been shown to contribute to biological processes with its anti-inflammatory, antioxidant and anticancer properties. These effects were demonstrated by focusing on the detrimental effects of IFO on lung tissue via oxidative stress and molecular pathways and by examining the potential protective role of morin on these pathways.

The mechanism of toxicity for many substances, including chemotherapeutic medicines, is based on oxidative stress.[24] Reactive oxygen species (ROS) or reactive nitrogen species rise as a result, causing tissue damage.[25,26] The increase of free radicals triggers lipid peroxidation by damaging lipids, which are essential components of the cell membrane.[27] MDA is the most prominent indicator of lipid peroxidation caused by oxidative stress.[28] Previous studies showed that MDA levels rose when IFO was administered and fell after antioxidant therapy was received.[12,29] In line with recent studies, our findings demonstrated that IFO administration led to elevated MDA levels, indicating enhanced oxidative stress and associated cellular damage.

IFO is metabolized by the cytochrome P450 3A4 enzyme, and the resulting metabolites are highly toxic. Cytotoxic nitrogen mustards with potent electrophilic qualities, acrolein, and chloroacetaldehyde are among these metabolites. GSH, an important antioxidant in cells, is rapidly depleted by isophosphoramide mustard, acrolein and chloroacetaldehyde, leading to toxic effects.[30] According to Çakmak et al., IFO caused oxidative stress in testicular tissue by inhibiting a number of antioxidant enzymes, such as SOD, CAT, and GPx, as well as non-enzymatic antioxidants, such GSH.[24] In another investigation, it was found that administering IFO raised MDA levels while lowering GSH levels.[31] In our study, it was determined that IFO application suppressed the antioxidant defense system by causing a decrease in SOD, CAT and GPx enzyme activities and GSH levels, and thus increased oxidative stress damage. Morin administered as a protective has been reported to alleviate oxidative stress with its antioxidant properties.[9,32] In the present study, morin was found to reduce oxidative stress-induced damage by increasing the activities of SOD, CAT, and GPx, as well as GSH levels, while decreasing MDA levels. Notably, morin administered at a dose of 200 mg/kg was observed to ameliorate oxidative damage more effectively.

Nrf-2 protects against oxidative damage and inflammation.[33] In healthy cells, Nrf-2 and kelch-like ECH-associated protein 1 (Keap1) form heterodimer. Nrf-2 and Keap1 separate when any damage is caused by different stimuli, and Nrf-2 is then carried to the nucleus. Once in the nucleus, HO-1 activates transport molecules, cellular antioxidants such as NADPH quinine oxidoreductase-1, glutamate-cysteine ligase modifier subunit and glutamate-cysteine ligase catalytic subunit.[34] A previous study by Han et al. reported that IFO administration down-regulated Nrf-2-mediated oxidative stress response pathways.[33] According to another study, chemotherapeutic agents decreased Nrf-2 expression.[35] In our study, it was observed that IFO suppressed Nrf-2 levels, reduced HO-1 expression in lung tissue, and consequently induced cellular damage due to oxidative stress. However, there is evidence that plant-derived compounds activate the Nrf-2/HO-1 pathway.[9,11] Our research revealed that the Nrf-2/HO-1 pathway, which is suppressed by IFO in lung tissue, is activated by morin, a plant-derived compound, and exhibits a protective effect against oxidative stress.

A crucial transcription factor, NF-κB controls inflammatory and immunological responses and enhances cell viability by shielding cells from apoptosis.[36] NF-κB is normally kept inactive in the cytoplasm by the inhibitory proteins IκB. However, after phosphorylation and proteasomal degradation of IκB, NF-κB is activated and translocated to the nucleus. There, it guides the immune response by promoting the production of cytokines that are essential for controlling the inflammatory response, like tumor necrosis factor (TNF-α) and interleukin-1β.[37] Research indicates that TNF-α is a cytokine that is essential for controlling the expression of iNOS in inflammatory situations.[38] Studies have reported that IFO causes inflammation by activating NF-κB and iNOS expression.[39,40] Bachewal et al. reported that morin administration markedly decreased NF-κB expression. Furthermore, it was reported that this decrease suppressed the inflammatory cascade together with the decrease in iNOS expression and thus morin provided a strong anti-inflammatory effect.[41] In the presented study, it was observed that IFO administration triggered the inflammatory response by increasing the expression of NF-κB and iNOS, whereas morin administration reduced inflammation by decreasing the expression levels of these molecules.

Apoptosis is a mechanism of cellular death triggered by increased levels of oxidative stress. Increased ROS levels trigger proapoptotic genes including NF-κB and TNF-α, which start the cell death process.[11,38] In this process, members of the Caspase family play a key role because they regulate cell death by being directly or indirectly involved in all stages of apoptosis.[42] The mitochondrial route is another pathway that contributes to apoptosis. Disruption of the Bax/Bcl-2 balance triggers the release of cytochrome c (CytC) from the mitochondrial space into the cytoplasm, initiating activation of the apoptosis pathway.[43] CytC, which enters the cytoplasm in the presence of adenosine triphosphate, binds to apoptotic protease activating factor-1 (Apaf-1) and activates it.[44] Activated Apaf-1 attaches itself to the cysteine aspartate specific protease-9 (Caspase-9) precursor form and causes it to become active. The apoptosis process is started when active Caspase-9 activates Caspase-3.[45] Bcl-2 exhibits an anti-apoptotic impact by preventing the activation of proapoptotic proteins, whereas Bax promotes apoptosis by eliminating growth factors.[9] IFO administration was found to initiate the apoptotic process by raising Bax and Caspase-3 levels while lowering Bcl-2 levels in a prior work on brain tissue.[5] In testicular damage, morin, a flavonoid, has been shown to decrease the expression of Bax and Caspase-3, increase the expression of Bcl-2, and prevent apaptosis.[46] In our current study, it was observed that Bcl-2 levels decreased and Caspase-3 and Bax levels increased in the lung tissue of rats exposed to IFO. These findings suggest that apoptosis may play an important role in IFO-induced lung injury. However, it was determined that morin administration had a protective effect in preventing mitochondrial apoptosis in rats exposed to IFO.

A cellular mechanism called autophagy uses lysosomes to eliminate aging and damaged organelles.[47] Beclin-1 and the anti-apoptotic protein Bcl-2 combine to inhibit autophagy.[48] However, if beclin-1 is overproduced or Bcl-2 levels are reduced, Bcl-2 cannot bind to beclin-1 and autophagy is activated.[13] According to a previous study, a significant increase in beclin-1 protein levels was detected in the testicular tissue of rats given IFO.[24] According to an in vitro study on chronic obstructive pulmonary disease, morin inhibited autophagy by inhibiting beclin-1 levels.[49] In our current study, in accordance with the literature, it was observed that IFO induced autophagy by increasing Beclin-1 levels, whereas morin administration provided a protective effect against autophagy by decreasing Beclin-1 levels.

As a result, this study shed light on the potential pathways behind the protective effects of morin on lung tissue in IFO-induced toxicity. Due to excessive ROS production and links to inflammation, oxidative damage, apoptosis and autophagy, IFO use causes lung toxicity. According to the findings of this study, simultaneous supplementation of morin provides a protective effect on the lung tissues of rats by reducing oxidative damage, inflammation, apoptosis and autophagy.

Ethics Committee Approval: The animal experimentation procedure was approved by the Animal Experiments Local Ethics Committee of Ataturk University Date: 31.01.2025, Approval No: 2025-31/26).

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