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Expression of IL-8 Gene in Rainbow Trout (*Oncorhynchus mykiss*) Leucocytes Fed with Uryani plum (*Prunus domestica*) Extract

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Abstract

Aim to study: In the present study, interleukin-8 (IL-8) gene expression was determined in rainbow trout (*Oncorhynchus mykiss*) after treatment with uryani plum (*Prunus domestica*) extract.

Material and methods: Fish leucocyte was stimulated with plum extract at the rate of 0 (control), 5 mg/ml, 10 mg/ml and 15 mg/ml. Cells were collected at 1, 4, 12, 24, and 48 hours of the study and IL-8 gene expression was determined in each group.

Results: The result showed an increase in IL-8 expression in all experimental groups compared to the control at the end of the study.

Conclusion: The results suggested that uryani plum (*Prunus domestica*) extract could activate immune responses of rainbow trout head kidney leukocytes in rainbow trout.

Keywords: Fish, gene expression, IL-8, immune response, *Prunus domestica*.

Üryani Eriği (*Prunus domestica*) Ekstraktı ile Beslenen Gökkuşağı Alabalığı (*Oncorhynchus mykiss*) IL-8 Geninin Ekspresyonu

Öz

Çalışmanın amacı: Bu çalışmada, üryani eriği (*Prunus domestica*) ekstraktı ile muameleden sonra gökkuşağı alabalıklarında (*Oncorhynchus mykiss*) interleukin-8 (IL-8) gen ekspresyonu belirlenmiştir.

Materyal ve yöntemler: Balık lökositleri; 0 (kontrol), 5 mg/mL, 10 mg/mL ve 15 mg/L oranında erik özütü ile uyarılmıştır. Hücreler çalışmanın 1, 4, 12, 24 ve 48. saatlerinde toplanmış ve her grupta IL-8 gen ekspresyonu belirlenmiştir.

Bulgular: Çalışma sonunda tüm deney gruplarında IL-8 ekspresyonunun kontrole kıyasla arttığı görülmüştür.

Sonuç: Sonuçlar, ince kabuklu erik (*Prunus domestica*) ekstraktının gökkuşağı alabalığı baş böbreği lökositlerinin immün yanıtlarını aktive edebileceğini göstermiştir.

Anahtar kelimeler: Balık, gen ifadesi, IL-8, immün yanıt, *Prunus domestica*.

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the most important coldwater fish species in aquaculture and is the 15th most produced species in the world. Global aquaculture production of rainbow trout has been increasing; according to Fishery and Aquaculture Statistics of Food and Agriculture Organization (FAO), global aquaculture production of rainbow trout was 916,540 tonnes in 2019 (FAO, 2022).

However, the emergence of various fish diseases has become one of the limiting factors in intensive aquaculture. Increasing stocking densities to produce more to meet growing demand has led to an increase in organic loading, which degrades water quality in the environment, and an imbalance in water parameters such as dissolved oxygen and pH, which are important for fish health. In addition, temperature (Bilen et al., 2013), poor water quality, and malnutrition trigger and manage the emergence of infectious diseases (Reverter et al., 2020). Chemotherapeutic agents, including antibiotics, provide an effective treatment option for disease outbreaks in aquatic animals; however, resistance has emerged due to their overuse against aquatic pathogens (Thanigaivel et al., 2016). In recent years, medicinal plants have been considered as feed additives for their growth-promoting, antioxidant, and immunostimulant activities. To minimise the use of antibiotics, the use of herbal immunostimulants in aquaculture is considered as one of the safest options. Herbal immunostimulants such as Greek juniper (*Juniperus excelsa*) aqueous methanolic extract (Bilen et al., 2021a), laurel leaf (*Cistus laurifolius*) ethanolic extract (Bilen et al., 2021b), black mustard (*Brassica nigra*) seed oil (Lakwani et al., 2021) and ribwort plantain (*Plantago lanceolata*) (Elbesthi, et al., 2020) were found to be effective against some important fish pathogens in fish.

On the contrary, *in vitro* studies evaluating the immunomodulatory effects of phytoextracts or phytochemicals on fish immune cells are still particularly scarce (Yin et al., 2006; Zanuzzo et al., 2012; Picchietti et al., 2013). Nevertheless, *in vitro* or *in vivo* approaches represent cost-effective pre-tests for subsequent *in vivo* experiments (Galeotti, 1998), in line with the 3Rs principle of replacement, reduction and refinement that should be applied to animal welfare (Midtlyng et al., 2011).

In vivo assays can be performed using primary cell cultures (e.g. leukocytes purified from lymphatic organs), whereas *in vitro* experiments use cell lines specifically developed for immunological research. These approaches provide reproducible results, allow simultaneous screening of many products at different concentrations and avoid the sacrifice of large numbers of fish (Fierro-Castro et al., 2012). In the case of *in vivo* tests, particular attention should be paid to the selection of healthy donors, as cell reactivity is influenced by the physiological status of the fish. Several promising immunostimulants such as glucans, lipopolysaccharide (LPS) or vitamins have been tested on fish immune cells (Mulero et al., 1998; Abarca et al., 2012; Fierro-Castro et al., 2012).

The main objective of the present study was to evaluate the effects of uryani plum (*Prunus domestica*) extract (TSP) on interleukin-8 (IL-8) expression in leukocytes purified from the head kidney of rainbow trout.

Material and Methods

Animals

The experiment was carried out on three rainbow trout (*Oncorhynchus mykiss* W., 1792) with an average weight of 30.61±1.09 g in Germeçtepe Inland and Marine Fish Production, Application and Research Center. Fish were adapted in

recirculating aquaculture systems in the Aquatic Toxicology unit of the Faculty of Fisheries for one week. For the present experiment, dried fruits were purchased from an herbal shop located in Kastamonu, Türkiye. The plum aqueous methanolic-extracts were prepared according to the method of Bilen et al. (2020).

Kidney Primary Cell Culture Medium

For primary culture, head kidney tissues of three rainbow trout were dissected under aseptic or sterile conditions, purified and maintained in a six-well plate and grown at 18 °C for at least 72 hours. Cell and tissue growth medium was Leibovitz's 15 (Gibco cat no. 11415064) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin (P/S) (Gibco, Thermo Fisher, Waltham, MA, USA) (Schnell et al., 2009).

***İn vitro* Immunostimulation**

24 h after seeding the explant for a primary kidney cell culture, it was stimulated with 0 (control), 5 mg/ml (TSP5), 10 mg/ml (TSP10) and 15 mg/ml (TSP15) concentrations of uryani plum. Primary cell cultures in six well plates were exposed to the immunostimulants for 1, 4, 12, 24 and 48 hours at 18 °C. At the end of these periods, the working cells were collected from each experimental well and harvested. Control plates contained the same volume of medium without immunostimulant. All experiments were performed in triplicate and independently repeated twice.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the samples using Direct-zol RNA MiniPrep extraction kits (Zymo, Cat. No. R2051) according to the manufacturer's instructions. Quantity and quality of RNA samples was checked using a Thermo Scientific Multiskan GO instrument at wavelengths of 260 and 280 nm. After qualitative measurements, RNA samples were synthesized into cDNA using an Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. cDNA reaction mixture contained 15 ng template RNA, 100 mM 25X dNTP mix, 10X RT random primers, 1 µL MultiScribe™ Reverse Transcriptase, 2 µL 10X RT buffer and 4.2 µL nuclease free water. The reaction mixture was incubated in a thermal cycler (ThermoFisher Scientific) for 10 minutes at 25 °C, 120 minutes at 37 °C and 5 minutes at 85 °C for cDNA synthesis and then stored at -20 °C.

Analyses of IL-8 Gene Expression

Primer sequences and references of the target genes are as indicated in Table.

Table. Gene specific primers with their sequences and references used for qRT-PCR in the study.

Gene	Primer sequence	References
β-actin	F: 5′-ATGGAAGGTGAAATCGCC-3′ R: 5′- TGCCAGATCTTCTCCATG- 3′	Sigh et.al. 2004
IL-8	F: 5′- CACAGACAGAGAAGGAAGGAAAG- 3′ R: 5′- TGCTCATCTTGGGGTTACAGA- 3′	Awad et. al. 2011

Expression levels of the genes were determined using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with the Wizpure qPCR Master SYBR Kit (Wizbio solutions, USA). The qRT-PCR mix contained 10 µL of 2× SYBR Green Master Mix, 1 µL template DNA (15 ng), 1 µL of each IL-8 forward and reverse primer, and distilled water to a final volume of 20 µL. The qRT-PCR protocol included 35 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 40 seconds, and extension steps. Fluorescence signals were captured at 530 nm wavelength from 60 °C to 95 °C, with a temperature increment of 0.5 °C per second, for melting curve analysis. Gene expression of the IL-8 was determined according to comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008). IL-8 gene expression in each sample was finally determined after correction with β-actin.

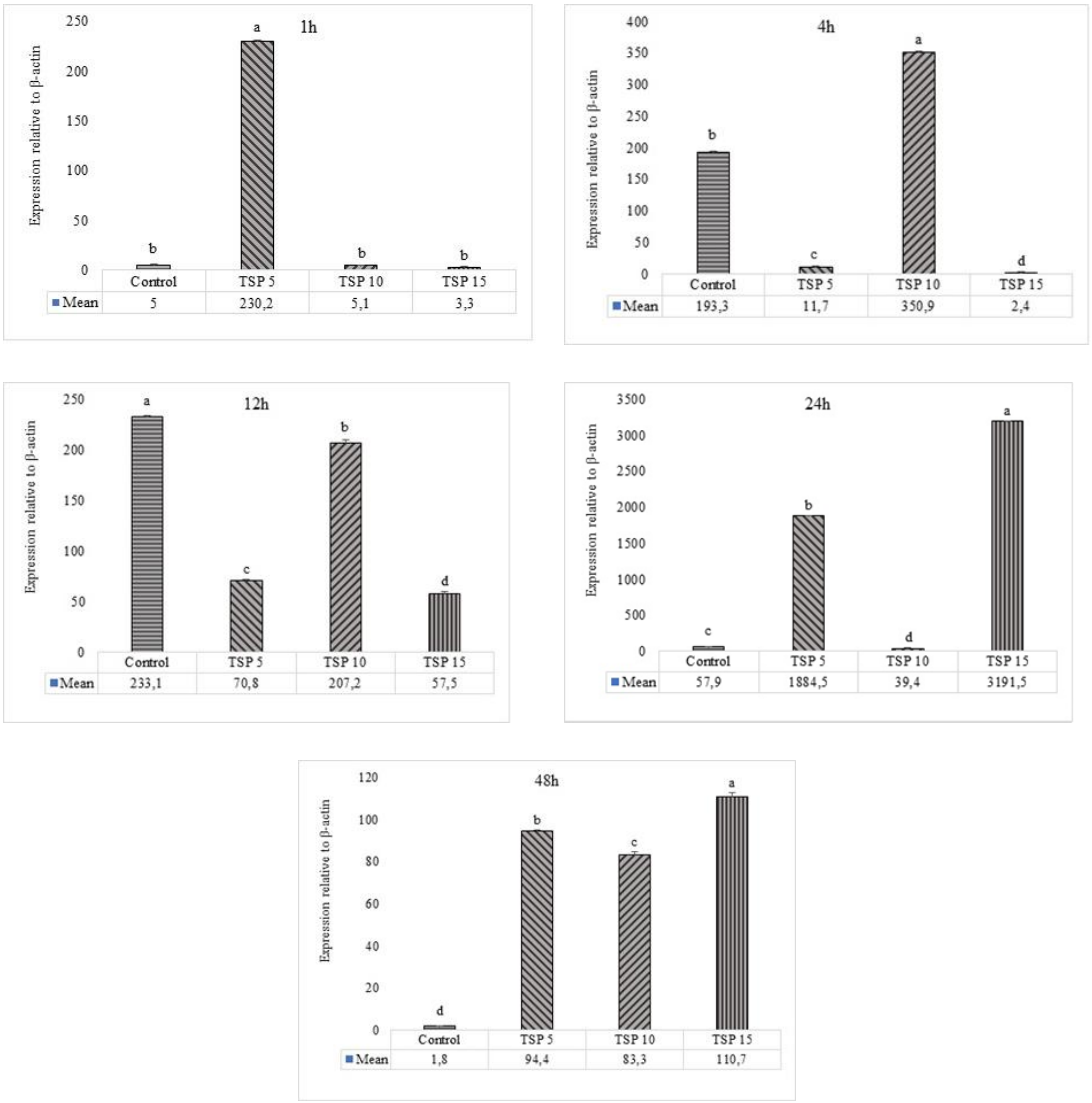

Figure. Comparison of relative gene expression levels (mean ± SD; n=3) of cytokines in head kidney cells of rainbow trout fed diets containing different doses of plum extract at the end of the 1st, 4th, 12th, 24th and 48th hours of feeding. Control, TSP5, TSP10 and TSP15 indicate uryani plum extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars denote significant differences among groups ($P<0.05$).

Statistical analyses

In our study, one-way analysis of variance (ANOVA) was performed to determine whether there was a statistically significant difference between the means of the dependent variables divided into different groups. The analysis was performed using SPSS 23.0 (SPSS Statistics) and the significance level was accepted as 0.05. Duncan's multiple comparison test was used when there was homogeneity of variances; otherwise, a Tamhane post hoc test was applied.

Results

The *in vitro* immunostimulatory effects of plum methanolic extracts at different concentrations were evaluated by changes in the expression levels of the IL-8 gene. At different sampling times, increases in IL-8 gene expression levels were detected in the experimental groups compared to the control group ($P<0.05$). IL-8 expression was found to increase in TSP5 group 1 hour after *in vitro* immunostimulant administration compared to the control group ($P<0.05$). However, at 4 h sampling time, this increase was observed in the TSP10 group ($P<0.05$).

As shown in the figure, IL-8 gene expression levels of the TSP5 and TSP15 groups were lower than the other groups at 12 hours ($P<0.05$). However, in contrast to this situation, as seen in the figure, TSP5 and TSP15 groups were the two groups with the highest gene expression levels compared to the other groups at 24 and 48 hours ($P<0.05$). When the effect of uryani plum extract on head kidney leukocyte of the rainbow trout stimulation *in vitro* was evaluated over 48 hours depending on the level of IL-8 gene expression, the highest effect was found in the TSP15 and TSP5 groups at 24 hours ($P<0.05$). Also, when the results were evaluated independently of the gene expression level, it was found that IL-8 expression in all experimental groups showed a significant increase compared to the control group at the end of 48 hours ($P<0.05$) (Figure).

Discussion

Pharmacological effects and immunomodulatory properties of plant extracts have been an important field of study for fisheries research (Bilen et al., 2016; Altunoglu et al., 2017; Elbesthi et al., 2020). In aquaculture, the immunostimulant effect of plant extracts has been widely investigated with humoral responses (Almabrok et al., 2018; Ali et al., 2022) as well as various cytokine genes and different results have been obtained (Salem et al., 2022; Sönmez et al., 2021; Terzi et al., 2021).

In the present study, IL-8 gene expression was determined at different sampling times in head kidney leucocyties of the rainbow trout. IL-8 is another important pro-inflammatory cytokine produced by a variety of cells including monocytes, macrophages, epithelial cells, endothelial cells, neutrophils, and fibroblasts (Jimenez et al., 2006). Under normal conditions, IL-8 is mainly distributed in the spleen, intestine and gill (Wang et al., 2017, Terzi te al., 2021). At the end of the study all groups IL-8 gene expression was significantly increased ($P<0.05$). IL-8, produced mainly by monocytes, can interact with the G protein-coupled receptors CXCR1 and CXCR2 to recruit neutrophils and induce cytotoxic effects at sites of infection (Kendrick et al., 2014).

Plum extract triggers the immune system and could probably reveal immune responses (Hooshmand et al., 2015). The results of this study clearly showed an increase in IL-8 and this increase was closely related to plum extract. The IL-8 is very important against gram negative bacteria (Wang et al., 2017). The methanolic extract of uryani plum showed an excessive increase in IL-8 gene expression. A sustained up-regulation of IL-8 cytokine was observed in the

kidney after 24 hours. Therefore, plum extract administration may have stimulated neutrophils and phagocytic cells. These results are in agreement with the findings of Salem et al. (2022) and Sönmez et al. (2021) who tested various plant immunostimulants. In contrast to our results, Altunoglu et al. (2017) were not able to detect any effect of black seed on the expression of the IL-8 gene in rainbow trout.

Conclusion

In conclusion, long-term use of uryani plum extract in rainbow trout may be an effective immunostimulant against bacterial diseases in rainbow trout. It is thought that uryani plum methanolic extract may be used not only as an immunostimulant but also as a remady agent.

Financial Support

This study did not receive a grant by any financial institution/sector.

Ethical Statement

This study was approved by the Kastamonu University Animal Experiments Local Ethics Committee (KUHADYEK-14.12.2020-2020.35).

Author Contributions

Investigation: S.B., N.C.A. and E.M.Y.; Material and Methodology: K.K. and N.C.A.; Supervision: S.B.; Writing-Original Draft: K.K. and E.M.Y; Writing- review & Editing: S.B.

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Comparison of Frozen Bull Spermatozoa After Direct Washing with The Brackett Oliphant Medium

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Abstract

Aim to study: The purpose of this study was to investigate the effects of the method of direct washing with the Brackett Oliphant (BO) medium which is used for purposes of *in vitro* fertilization on some spermatological parameters in Holstein and Brown Swiss bulls.

Material and methods: The study used cryopreserved sperm obtained from the Holstein (n=5) and Brown Swiss (n=5) breeds. After this procedure, Computer Assisted Sperm Analysis (CASA) and oxidative status analyses were conducted both before and after direct washing method with BO. The data were analyzed using the statistical methods of paired-samples t-test and independent-samples t-test.

Results: After direct washing with BO, the total motility in the Brown Swiss group decreased from 62.67 to 33.33 (P=0.011). After washing with BO, the total antioxidant level decreased from 1.45 to 0.11 in Group 1 (P = 0.000) and from 0.83 to 0.09 in Group 2 (P=0.000). Additionally, the total oxidant level increased from 5.54 to 5.70 in Group 1 (P = 0.024) and from 4.94 to 5.12 in Group 2 (P=0.019).

Conclusion: According to the findings, the direct washing method with BO can negatively affect Brown Swiss spermatozoa motility. Additionally, after washing, the antioxidant level significantly decreases, and the oxidant levels increase due to oxidative stress.

Keywords: Brackett-Oliphant, CASA, frozen bull semen, oxidative status, spermatozoa.

Dondurulmuş Boğa Spermasının Brackett Oliphant Medyumu ile Doğrudan Yıkama Sonrası Karşılaştırılması

Öz

Çalışmanın Amacı: Bu çalışmanın amacı, *in vitro* fertilizasyon amaçlarıyla kullanılan Brackett Oliphant (BO) ortamı ile doğrudan yıkama yönteminin Holstein ve Brown Swiss boğalarında bazı spermatolojik parametreler üzerindeki etkilerini incelemektir.

Materyal ve Yöntemler: Çalışmada, Holstein (n=5) ve Brown Swiss (n=5) boğalarından elde edilen dondurulmuş spermatozoalar kullanıldı. Prosedür sonrasında, Bilgisayar Destekli Sperm Analizi (CASA) ve oksidatif stres analizleri, doğrudan BO ile yıkama yöntemi öncesinde ve sonrasında gerçekleştirildi. Veriler, çift örneklem t-testi ve bağımsız örneklem t-testi istatistiksel yöntemleri kullanılarak analiz edildi.

Bulgular: BO ile doğrudan yıkama sonrasında, Brown Swiss grubunda toplam motilite 62,67'den 33,33'e düştü (P=0,011). Grup 1'de BO ile yıkandıktan sonra toplam antioksidan seviyesi 1,45'ten 0,11'e düşerken (P=0,000), Grup 2'de 0,83'ten 0,09'a düştü (P=0,000). Ayrıca, Grup 1'de toplam oksidan seviyesi 5,54'ten 5,70'e yükseldi (P=0,024) ve Grup 2'de 4,94'ten 5,12'ye yükseldi (P=0,019).

Sonuç: Elde edilen bulgulara göre, BO ile direkt yıkama yöntemi spermatozoa hareketliliğini olumsuz yönde etkileyebilir. Bu etki farklı ırklarda aynı şekilde ortaya çıkmamaktadır. Ayrıca, yıkama işleminden sonra antioksidan seviyesi önemli ölçüde azalırken, oksidan seviyeleri oksidatif stres nedeniyle artmaktadır.

Anahtar kelimeler: Brackett-Oliphant, CASA, dondurulmuş boğa sperması, oksidatif durum, spermatozoa.

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Introduction

Cryopreserved bull spermatozoa are generally used for *in vitro* embryo production. For this purpose, several sperm preparation methods have been developed, including Swim-up, Percoll gradient, Filtration or Direct Washing with Brackett Oliphant (BO) (Gordon, 2003). In some methods used for *in vitro* embryo production, the seminal plasma, which protects against negative effects like oxidative stress, is removed, a procedure is called Sperm Washing (Saleh & Ashok, 2002; Martí et al., 2006). Sperm preparation methods are selected based on their characteristics of being economical and easy to prepare, yielding high numbers of motile spermatozoa after the procedure, causing minimal damage to the spermatozoa during the procedure, and easiness in removal of other cells and decapacitation factors or substances that lead to oxidative stress (Henkel & Schill, 2003). Brackett & Oliphant (1975) showed that with the High Ionic Strength (HIS) medium they obtained by adding a sufficient quantity of sodium chloride into the BO medium, they stimulated sperm capacitation and removed factors that induce decapacitation from the spermatozoa membrane, and they obtained successful results in their studies on *in vitro* capacitation of mouse and rabbit sperm. However, it was emphasized that a loose penetration was obtained in bull spermatozoa with the HIS medium, and this may be related to the bull from which the spermatozoa were obtained (Parrish, 2014). Computer-Assisted Sperm Analysis (CASA) provides quantitative information on spermatozoa and has GFHB been used for a long time to determine spermatological parameters and spermatozoa-related infertility detection. With the help of this technology, it is easy and effective to collect values that are called Total Motility (TM), Progressive Motility (PM), Curvilinear Velocity (VCL), Straight Line Velocity (VSL) and Velocity Average Pathway (VAP), and the value of VCL is accepted as a significant indicator of spermatozoa vitality (Verstegen et al., 2002).

Oxidative stress is a significant parameter which is an indicator of spermatozoa quality, and it is analyzed by several laboratories as a major factor for determining male infertility (Saleh & Ashok, 2002; Robert et al., 2021). It was reported that the Total Antioxidant Capacity (TOC) in the seminal plasma of fertile males was higher than that of infertile males (Lewis et al., 1995). Additionally, the pathological level of Reactive Oxygen Species (ROS) found in infertile males may be more effective on infertility in comparison to low antioxidant capacity (Zini et al., 1993). Motility loss and DNA damage in sperm nuclei can also occur due to oxidative stress caused by Lipid Peroxidation (LP) (Saleh & Ashok, 2002). Some intracellular and extracellular mechanisms that prevent oxidative stress work in steps of prevention, interception and repair. However, as the cytoplasmic enzyme systems of spermatozoa are not on an adequate level, they are highly vulnerable to oxidative stress (Sies, 1993).

The BO washing method largely separates the seminal plasma from the sperm. As a result, antioxidants in the seminal fluid may be removed from the sperm, potentially affecting oxidative stress and thus sperm parameters. This study investigated the changes in sperm oxidant and antioxidant levels after direct washing with BO and the possible effects of the method on sperm motility.

Material and Methods

Animals and Semen Collection

This study used frozen spermatozoa obtained from 10 different bulls used by the International Livestock Research and Training Center (39º58’07.49” N, 33º06’29.86” E – Altitude: 1079 m) as artificial insemination bulls. Group 1 (G1) consisted of Holstein (n=5) and Group 2

(G2) consisted of Brown Swiss (n=5) spermatozoa which were frozen and kept in liquid nitrogen. The spermatozoa were collected in the same week, diluted in AndroMed (Minitube/Germany) and frozen based on the standard freezing protocol of the laboratory. The protocol is briefly used involved diluting the sperm with AndroMed solution to a concentration of 60×10^6 / ml spermatozoa at room temperature. After incubating for 4 hours at +4 °C, the samples were transferred into straws (IMV, France) with a volume of 0.25 ml using a Cold Handling Cabinet (IMV, France). Subsequently, the straws were exposed to liquid nitrogen vapor for 10 minutes and then plunged into liquid nitrogen. Attention was paid to making sure there was at least 55% subjective motility in the last checks on the spermatozoa in 0.25 ml commercial straws containing 175×10^5 spermatozoa, and the groups were formed by random sampling after the thawing procedure (Ansari et al., 2017).

The sperm was thawed in a 37 °C water bath for 30 seconds, then transferred to 15 ml plastic centrifuge tubes. For this purpose, BO medium was added to sperm at a ratio of 5:1. The sperm was centrifuged at 1000 G for 5 minutes. After centrifugation, the supernatant was removed, and the pellet was resuspended in BO medium again. This process was repeated once more to prepare the sperm for CASA and oxidative parameter analysis. The contents of the BO solution are specified in Table 1 (Kanagawa et al., 1995).

CASA Analysis

For each bull, at least 10 straws were diluted, and analyzed in terms of CASA (IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) and Total Antioxidant and Oxidant Capacity. Briefly, for CASA analysis, approximately 3 µl of sperm are placed on a Leja slide (Leja 4; IMV, France) and inserted into the device's sample compartment. Once the appropriate focus is achieved, the analysis is conducted, and the results (Total Motility (%), Progressive Motility (%), VAP (µm/s), VSL (µm/s) and VCL (µm/s)) are recorded. After washing the spermatozoa with the method of direct washing with the BO medium in a short time, CASA and oxidative measurements were carried out without wasting time, and all the procedures were repeated at least two times for each bull.

Table 1. Content of Bracket Oliphant Medium

Ingredient	Quantity
NaCI	0.6549 g
KCI	0.0300 g
$CaCI_2 2H_2O$	0.0329 g
$NaH_2PO_4 2H_2O$	0.0127 g
$MgCI_2 6H_2O$	0.0105 g
$NaHCO_3$	0.3104 g
Sodium Pyruvate	0.0138 g
Penicilin	10.000 IU
Streptomycin	10 mg
Caffeine	0.3884 g
Heparin	0.1 ml
%0.05 Phenol Red	20 µl
Deionized Water	100 ml

The components and quantities of the solution are provided for informational purposes. For details on the preparation of the solution, refer to the study conducted by Kanagawa et al. (1995).

Oxidative Stress Parameters

Oxidative stress measurements were made by the method described by commercial Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) Test Kits (Real Assay Diagnostics® Mega Tıp, Gaziantep / Türkiye) based on the enzyme-linked immunosorbent assay (ELISA) method.

Statistical Analysis

The SPSS V15 (SPSS Inc., Chicago, IL, USA) software was used for statistical analyses. The data are presented as means and standard deviations. The level of significance was selected as $P \leq 0.05$. Paired-samples t-test was used for the after-thawing and post-BO data, while

independent-samples t-test was used to compare differences between the groups.

Results

Progressive motility, VAP, VSL and VCL values differed among neither the Holstein nor the Brown Swiss breeds (P>0.05). Total motility decreased significantly in the spermatozoa of the Brown Swiss breed after treatment with the BO medium (P≤0.05). BO treatment didn't cause a significant change in the spermatozoa of the Holstein and Brown Swiss breeds. Likewise, no significant differences were observed in the pre-BO and post-BO treatment VAP/VSL/VCL values (Table 2).

Table 2. Evaluation of bull semen with computer assisted semen analysis results

Items	**Before Washing with Bracket-Oliphant Medium**	**After Washing with Bracket-Oliphant Medium**	**P Value**
	G1 (mean ± SD)		
Total Motility (%)	46.0 ± 6.08	41.33 ± 13.61	0.109
Progressive Motility (%)	16.67 ± 3.21	11.67 ± 6.51	0.066
VAP (μm/s)	82.33 ± 19.45	73.17 ± 16.98	0.094
VSL (μm/s)	64.27 ± 9.74	62.57 ± 14.40	0.085
VCL (μm/s)	142.43 ± 42.39	128.5 ± 24.73	0.072
	G2 (mean ± SD)		
Total motility (%)	62.67 ± 18.88	33.33 ± 23.63	0.011
Progressive motility (%)	18.67 ± 9.24	11.33 ± 9.29	0.065
VAP (μm/s)	77.33 ± 8.57	83.7 ± 6.58	0.082
VSL (μm/s)	58.2 ± 3.44	70.77 ± 7.39	0.071
VCL (μm/s)	134.43 ± 15.88	146.53 ± 19.08	0.066

P-value of ≤ 0.05 assumed as significant.

As expected, after the process of BO treatment, in the spermatozoa of both the Holstein and Brown Swiss breed of bulls, the TAS values that are the indicator of antioxidant capacity decreased (P≤0.05), and similarly, the TOS values that are the indicator of oxidant capacity increased (P≤0.05). There was no difference between the breeds in terms of antioxidant products or oxidative products (Table 3).

Table 3. Antioxidative and oxidative status of between two breeds

Groups	Total Antioxidan Status (mean ± SD) (mmolTrolox Equiv./L)			Total Oxidan Status (mean ± SD) (mmolTrolox Equiv./L)		
	Before washing with Bracket-Oliphant medium	After washing with Bracket-Oliphant medium	P Value	Before washing with Bracket-Oliphant medium	After washing with Bracket-Oliphant medium	P Value
G1	1.45 ± 0.34	0.11 ± 0.03	0.000	5.54 ± 1.03	5.70 ± 1.05	0.024
G2	0.83 ± 0.05	0.09 ± 0.02	0.000	4.94 ± 0.73	5.12 ± 0.88	0.019

P-value of ≤ 0.05 assumed as significant.

Discussion

According to the findings, washing with BO has been found to reduce sperm antioxidant levels and increase oxidant levels (P≤0.05). Seminal plasma contains several antioxidant enzymes, including catalase, glutathione peroxidase and superoxide dismutase (Tvrdá et al., 2013). It is believed that centrifugation of sperm reduces the levels of these enzymes, and the resulting mechanical stress triggers oxidative stress in sperm. Mechanical stress has been shown to increase oxidative stress in sperm of many species (Agarwal et al., 2009; Dominiguez-Robelledo et al., 2009; Sarıözkan et al., 2010). This increase in reactive oxygen species due to oxidative stress damages the sperm cell membrane with lipid peroxidation. Therefore, the resulting cellular damage affects spermatozoa motility (Kurkowska et al., 2020) According to researchers, spermatozoa with VCL values higher than ≥ 70 are hyperactive (Verstegen et al., 2002). Therefore, it was understood in our study that the VCL values were high, the bull spermatozoa in all groups were hyperactive. It is possible to say that the BO washing method does not contribute to this situation. However, it is believed that the capacitatation effect could be due to the heparin present in the BO medium (Parrish, 2014). It was observed that progressive motility was lower than expected among all the spermatozoa. The post-thawing motility in all groups was lower than the findings of several studies (Lee et al., 2009; Orgal et al., 2012; Bucak et al., 2015; Tırpan et al., 2017). Total motility in the sperms of the Brown Swiss bull breed decreased significantly after the BO treatment process (P≤0.05). This might have been caused by individual factors. The resistance of the spermatozoa of the Holstein bulls against freezing differs as lipid transport is different among males (Waterhouse et al., 2006). Therefore, it is expected that there is such a difference among breeds and individuals in such studies. It was observed in both breeds that post-thawing antioxidant product levels decreased, and after the BO treatment, oxidative product levels increased (P≤0.05). It is expected that antioxidant levels drop after BO treatment because most of the substances in sperm diluents that have antioxidant properties are removed from the environment along with the supernatant. Similarly, it is also expected that the quantity of oxidative products in the environment will increase based on the reduction of antioxidant substances in the medium. Chaveiro et al. (2007) study used the swim-up method and reported an increase in the membrane destabilization of spermatozoa. In spermatozoa processing methods and during freezing and thawing processes like

this, multiple membranous changes lead to reductions in spermatozoa motility and capacity, and/or acrosomal reaction (Medeiros et al., 2002). Thus, it is a natural outcome that sperm washing processes carried out after thawing processes create effects that may lead to exposure of sperms to oxidative stress in *in vitro* environments and disruption of their membrane integrity, and this situation acts against spermatozoa as the waiting period of sperms increases.

It may be stated that the post-thawing quantity of oxidative products in this study was high. This may be explained by the low levels of protection of spermatozoa by sperm diluents. Although Aires et al. (2003) stated that non-animal-based diluents that use soy lecithin are good alternatives, it is known that the lecithin in egg yolks has a high capacity to protect spermatozoa. Researchers consider high levels of oxidative stress as an indicator of spermatozoa-related infertility (Saleh & Ashok, 2002).

Conclusion

As a result, it was observed that the process of sperm washing using the BO medium for purposes of *in vitro* fertilization purposes provided similar results in different breeds. However, the results indicate that the primary effect of the BO medium on capacitation may be primarily due to its heparin content. It is observed that the solution does not have a direct effect on hyperactivity markers. Therefore, future studies may need to develop an alternative method to centrifugation, which is known to negatively affect sperm. If centrifugation is to be used, future research could determine whether the antioxidant deficit resulting from centrifugation can be compensated for by adding external antioxidants to the diluent.

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Ethical Statement

As the study material was commercially available and no live animals were used for the experiments, ethics committee approval was not required.

Author Contributions

Investigation: N.A. and A.K.; Material and Methodology: N.A. and A.K.; Supervision: A.K.; Writing-Original Draft: N.A.; Writing- review & Editing: A.K**.**

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Cabergoline Administration after Ovariohysterectomy in a Queen with Fibroepithelial Hyperplasia

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Abstract
In this case report, the diagnosis and treatment process of fibroepithelial hyperplasia developing in the mammary glands of an 11-month-old Tabby queen is presented. Anamnesis that the queen had been in estrus once before, it mammaries were gradually swollen in the last 15 days, her appetite and general condition were normal, and there was no previous progesterone administration. On physical examination, all mammary lobes were excessively tight bilaterally, swollen mammaries were gel-like and painless on palpation, hyperemia and milk secretion around the nipples were noted. Laboratory tests showed that serum progesterone level was 3.02 ng/mL and estrogen level was 5.2 pg/mL. Fibroepithelial hyperplasia was diagnosed based on anamnesis, clinical examination, and laboratory analysis. Ovariohysterectomy operation was performed as the treatment. One day after the operation, cabergoline was administered orally at a dose of 5 µg/kg/day for 5 days. At the two weeks-follow-up examination, a significant regression in the mammary glands size and a end of milk production were observed. In conclusion, fibroepithelial hyperplasia in the mammary glands of a young queen that was brought to our clinic were successfully cured endogenously by surgical method and exogenously by medical drug administration.
Keywords: Cabergoline, fibroepithelial hyperplasia, ovariohysterectomy, queen.

Fibroepitelyal Hiperplazili Bir Kedide Ovaryohisterektomi Sonrası Kabergolin Uygulaması

Öz
Bu olgu sunumunda 11 aylık dişi tekir kedinin meme bezlerinde gelişen fibroepitelyal hiperplazinin tanı ve tedavi süreci sunulmaktadır. Anamnezde kedinin daha önce bir kez kızgınlık geçirdiği, son 15 gündür memelerinin giderek şiştiği, iştahının ve genel durumunun normal olduğu, daha önce progesteron uygulaması olmadığı öğrenildi. Fiziksel muayenede tüm meme loblarının iki taraflı aşırı gergin olduğu, memelerinin şişmiş, jel kıvamında ve palpasyonda ağrısız olduğu, meme başı çevresinde hiperemi ve süt salgısının olduğu görüldü. Laboratuvar testlerinde serum progesteron düzeyi 3,02 ng/mL, östrojen düzeyi ise 5,2 pg/mL olarak belirlendi. Fibroepitelyal hiperplazi tanısı anamnez, klinik muayene ve laboratuvar analizine dayanarak konuldu. Tedavi olarak ovaryohisterektomi operasyonu uygulandı. Operasyondan bir gün sonra kabergolin 5 gün süreyle 5 µg/kg/gün dozunda oral olarak uygulandı. İki haftalık kontrolde meme bezlerinin boyutunda belirgin bir gerileme ve süt üretiminin sona erdiği görüldü. Sonuç olarak kliniğimize getirilen genç bir dişi kedinin meme bezlerinde oluşan fibroepitelyal hiperplazi oluşumunun endojen olarak cerrahi yöntemle, eksojen olarak ise medikal ilaç uygulamasıyla başarılı bir şekilde iyileştirildiği görüldü.
Anahtar kelimeler: Kabergolin, fibroepitelyal hiperplazi, ovaryohisterektomi, dişi kedi.

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Introduction

Feline mammary fibroepithelial hyperplasia (MFH) is a condition marked by the rapid, non-cancerous growth of both the ductal epithelium and stroma within the mammary glands, leading to the enlargement of one, multiple, or all of the glands (Allen, 1973). This condition usually occurs during first estrus (5-12 months), pregnancy or pseudopregnancy. It is often seen in young queens (13 weeks to 2 years old). Cases of MFH can also be seen in male and queens of all ages that receive exogen progesterone compounds such as megestrol acetate (MA) and medroxyprogesterone acetate (MPA) to suppress estrus (Görlinger et al., 2002).

The affected mammary glands of the queen with fibroepithelial mammary hyperplasia is soft, sharply limited, fluctuant, and jelly-like. Furthermore, it has been reported that some patients have erythematous, dark, and necrotic areas on the their skin of the relevant mammary gland (Bonatto et al., 2021; Voorwald et al., 2021). Fever, tachycardia, lethargy, anemia, and anorexia can be seen as systemic effects of the disease (Loretti et al., 2004). Although fibroepithelial hyperplasia can be diagnosed commonly by clinical findings (inflammation of the mammary gland, etc.), the homogeneous and granular structure of the parenchyma of the affected mammary glands can be seen characteristically during ultrasonographic examination. In addition, histopathological examination of fine needle aspiration biopsy or excision biopsy specimens is also recommended as an additional diagnostic technique (Vitasek & Dendisova, 2006). Treatment can be done by using drugs with luteolytic effect and/or ovariectomy, ovariohysterectomy (OHE), and other methods to prevent endogenous progesterone effect (Johnston et al., 2001; Keskin et al., 2008). Treatment options include surgical methods such as total and partial mastectomy, however, it can be successfully treated with the application of progesterone receptor blockers as medical treatment (Görlinger et al., 2002).

In this case report, the diagnosis of fibroepithelial hyperplasia in the queen and the rapid and nonrecurrent treatment process are presented.

Case Description

The study material consisted of an 11-month-old tabby queen, weighing 4 kg, which was brought to the Animal Hospital of the Faculty of Veterinary Medicine of Harran University. Anamnesis taken from the patient's owner revealed that the queen had been in heat once before, her mammaries had been gradually swollen in the last 15 days, and her appetite and general condition were normal. The queen was fed a commercially available dry diet, vaccinated regularly, and no medication use (steroid, antifungal or progestin) was reported in her anamnesis.

Physical examination revealed that the queen's body temperature (38.7 ^{o}C), pulse (114/min), and respiration (14/min), were within normal limits. It was observed that the mammary lobes were extremely tight, the swollen mammaries were gel-like and painless on palpation, and there was also milk secretion (Figure). Furthermore, it was noted that the swellings did not have a connection with the abdominal wall. In the complete blood count analysis (pocH-100 IV Diff, Sysmex, Norderstedt, Germany), all parameters (WBC: 6.2 x 10^3/µL, RBC: 9.7 x 10^6/µL, HGB: 13.0 g/dL, HCT: 41.4 %, MCV: 40.6 fL, MCH: 14.7 pg, MCHC: 30.1 g/dL, PLT: 206 x 10^3/µL) were found to be within normal limits. Serum progesterone level was 3.02 ng/mL and estrogen level was 5.2 pg/mL. Based on the medical history, clinical examination, and serum progesterone level, the diagnosis of MFH was made.

Figure. Appearance of the mammary glands before treatment.

In the treatment, an ovariohysterectomy operation was performed through a median line incision. During the operation, corpus luteums that had become cystic on the ovary were observed. Antibiotic (containing 140 mg amoxicillin trihydrate and 35 mg clavulanic acid per ml, Synulox®, Zoetis New York, USA) and vitamin (Nervit® composition/Vetaş, Türkiye) support was given for 5 days after the operation. One day after the operation, cabergoline (Dostinex®, Pfizer, New York, USA) was administered orally at a dose of 5 µg/kg/day once a day for 5 days. In the follow-up examination two weeks later, a significant regression of the mammary glands and a end of milk production were observed. Follow-up examination performed one month later revealed that the mammary glands were completely recovered and the serum progesterone value was 0.217 ng/mL and the estrogen value was less than 5 pg/mL. No complications were encountered during and after the treatment.

Discussion

Fibroepithelial hyperplasia is formed due to the hypersensitivity of mammary gland tissues to progesterone during the estrus period of the reproductive cycle. This problem spontaneously disappears in a long period of 40-45 days following the natural regression of the corpus luteum (Loretti et al., 2004). However, as waiting for this process can result in several adversities in the mammary glands, this process should be accelerated by early intervention (Allen, 1973; Görlinger et al., 2002). Although fibroepithelial hyperplasia is generally seen in young queens (Baştan et al., 2004; Uçmak et al., 2011), cases can also be observed in male and female cats of all ages who are administered exogenous synthetic progestins for a short or long time (Görlinger et al., 2002; Nak et al., 2004; Bonatto et al., 2021). Similar to the report of Görlinger et al., (2002), a case of MFH was encountered in a queen at a very early age without any treatment, therefore, estrogen and progesterone hormones of individual mammary glands were encountered, suggesting that MFH may have occurred as a result of increased susceptibility due to factors such as genetics and age.

In queens with fibroepithelial mammary hyperplasia, increases in tissue volume lead to perfusion problems, erythema, pain, ulceration, dark and necrotic areas on the mammary gland skin (Bonatto et al., 2021; Voorwald et al., 2021), as well as systemic effects such as high fever, tachycardia, anorexia, and sepsis are also observed (Loretti et al., 2004; Voorwald et al., 2021). In this case, growth was observed to cover all mammary lobes, similar to other researchers (Baştan et al., 2004; Vitasek & Dendisova, 2006; Küçükbekir et al., 2020), and there were no local and general symptoms except hyperemia in some mammary lobes. It was assumed that this might be since the queen was not exogenously exposed

to progestins, and therapeutic intervention was commenced in the clinic in a short time.

In a study where aglepristone (15 mg/kg/ 2-3 weeks) and cabergoline (5 µg/kg/oral/ 7 days) were administered together, it was found that the size of the mammary glands was reduced significantly and milk secretion was ceased at the 3rd week, and the mammary glands were completely regressed after 6 weeks (Uçmak et al., 2011). In another study, it was reported that the treatment with aglepristone and cabergoline (5µg/kg/14 days) in combination was effective in stopping milk production and ensure regression in the mammary glands in a pregnant queen with mammary gland hyperplasia (Keskin et al., 2008). When the hyperplastic change in the mammary gland is due to an endogenous source of progesterone, ovariohysterectomy is one of the most effective treatment methods, as suggested by other researchers (Baştan et al., 2004; Kutzler & Wood, 2006; Silva & Silva, 2012). When making this decision, it should also be taken into consideration that this is an operation that will prevent it from being possible to have offspring in the future (Melo et al., 2021). Baştan et al. (2004) reported that in a case of mammary hypertrophy observed in a 5-month-old queen, all mammary glands, except the mammary gland where hypertrophy started, returned to normal after the follow-up performed only on the 7th day after the OHE operation, and clinical improvement was observed in all mammary glands on the 14th day. However, in a study in which the effect of OHE was seen on MFH, it was reported that persistent swelling of the mammary gland was encountered more frequently after the operation, and treatment with antiprogestogen would provide a better success in these complicated cases (Melo et al., 2021). In our study, the combination of OHE and cabergoline was administered to the mammary glands in two different ways (Nak et al., 2004; Uçmak et al., 2011; Küçükbekir et al., 2020). A significant regression was noted within a week and no complication related to both the operation and the regression process in the mammary gland was observed. It was thought that the success in this rapid regression period in the mammary glands might be related to the combination of treatment and the fact that medications such as MPA were not used in the anamnesis. Furthermore, it is stated that OHE performed through the median incision before the complete involution of the mammary glands is impossible, and it should be performed after the complete involution of the hyperplasia (Munson, 2006), and no operative complication was encountered in this presented case. The dose of cabergoline administered in this case, especially in which there was milk secretion, was consistent with other study data (Keskin et al., 2008; Uçmak et al., 2011), and it was seen that cabergoline administration at the specified dose did not cause any adverse events. Contrary to some studies (Keskin et al., 2008; Uçmak et al., 2011), the administration time was kept short, limited to 5 days in terms of supporting OHE operation and not for therapeutic purpose. It was proved to be successful. It has been stated that the regression process of the mammary gland is rather long, especially in cases where short or long-term progestagen was used in the past and in those receiving only medical treatment. The mammary lobes become ulcerated and relapses occur shortly after the application in those cases (Keskin et al., 2008; Uçmak et al., 2011). Therefore, it has been observed that combined treatment, including surgery, is an effective treatment method in MFH cases that do not respond to treatment.

Conclusion

As a result, fibroepithelial hyperplasia was successfully treated and possible recurrences were avoided through OHE operation, suppressing milk secretion with cabergoline and accelerating mammary glands regression,

especially in queens that are not planning to become pregnant.

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Ethical Statement

This study does not present any ethical concerns.

Author Contributions

Investigation: T.A. and Ö.Y.; Material and Methodology: T.A. and Ö.Y.; Supervision: T.A. and Ö.Y.; Visualization: Ö.Y.; Writing-Original Draft: T.A. and Ö.Y.; Writing- review & Editing: T.A.

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Successful Treatment of Olecranon Fracture with Tie-in External Fixator in a Cat

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Abstract

In this case report, a 4-month-old male British shorthair cat was brought with lameness, swelling in the forelimb, and loss of appetite following a fall from a height. On the orthopedic examination,pain, lameness, abnormal mobility, local sensitivity, crepitation, and deformation findings were observed in the forelimb. Clinical and radiologic examinations revealed a fracture of the proximal left olecranon. The fracture was treated with an external fixator (ESF). Clinical and radiological controls were performed on the 10th, 21st, 30th, and 45th postoperative days. The External fixator was removed on the 62nd postoperative day under sedation. This case report aimed to contribute to the literature by presenting the successful treatment of an olecranon fracture in a 4-month-old young cat using an ESF.

Keywords: Cat, cubiti, elbow joint, feline, ulna.

Bir Kedide Olecranon Kırığının Tie-in Eksternal Fiksatör ile Başarılı Sağaltımı

Öz

Bu olgu sunumunda, 4 aylık, erkek, British shorthair ırkı bir kedi yüksekten düşme sonrası ön bacakta topallık, şişlik ve iştahsızlık şikayetleri ile getirildi. Ortopedik muayenede ön ayakta ağrı, topallık, anormal hareketlilik, lokal hassasiyet, krepitasyon ve deformasyon bulguları gözlendi. Klinik ve radyolojik incelemelerde sol olekranonda avülsiyon kırığı tespit edildi. Kırık eksternal fiksatör ile tedavi edildi. Ameliyat sonrası 10, 21, 30 ve 45. günlerde klinik ve radyolojik kontroller yapıldı. Eksternal fiksatör ameliyat sonrası 62. günde sedasyon altında çıkarıldı. Bu olgu sunumunda, 4 aylık genç bir kedide olekranon kırığının eksternal fiksatör ile başarılı tedavisi konusunda literatüre katkıda bulunulması amaçlandı.

Anahtar kelimeler: Kedi, cubiti, dirsek eklemi, ulna.

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Introduction

The olecranon is the most proximal part of the ulnar bone and consists of the tuber olecranon, the anconeal process, and the proximal part of the trochlear articular notch. The triceps brachii muscle adheres to the olecranon process of the ulnar bone and helps the elbow joint to extend and bear the weight of the extremity (Paul et al., 2021).

Olecranon fractures can be extra-articular or intra-articular and in both cases, the bone fragment is displaced proximally by the triceps muscle pull. This type of fracture is usually seen in young dogs. Olecranon fracture requires special attention and extra care; if not treated appropriately, it may result in delayed nonunion, often leading to functional disuse of the limb. The main problem with this fracture is that the fractured segments are displaced by the traction of the triceps muscle (Şen & Sağlam, 2012; Şen et al., 2015; Sarıerler & Kibar Kurt, 2021) .

Olecranon fracture can be repaired by applying different techniques such as pin and external fixation or tension band or tension band and lag screw combination or Kirschner wire (K- wire) and tension band combination. Among these, the tension band technique is commonly used in the repair of olecranon fractures and provides adequate stabilization as well as immediate weight-bearing of the limb. The basic principle of tension banding is to counteract the tensile forces acting along the fracture site and convert them into compressive forces (Paul et al., 2021).

The application of external skeletal fixator (ESF) made of lightweight epoxy material rods for fracture repair is used in veterinary orthopedics. Lower cost, mechanical durability, greater versatility, reduced operating time, and simplified application techniques have made epoxy external fixators increasingly preferred over both internal and external fixation techniques (Kurt & Sen, 2022). The aim of this study was to present an alternative method for the intervention of olecranon fractures with the application of tie-in type external fixation, which has not been previously applied in cat proximal olecranon fractures.

Case Description

A 4-month-old, 1.75 kg male British Shorthair cat was brought to Aydın Adnan Menderes University Faculty of Veterinary Medicine, Department of Surgery, with a complaint of lameness in the left forelimb. In the anamnesis, it was learned that the patient had fallen from a height 2 days ago. The first emergency intervention was performed in a private veterinary clinic immediately after the fall. Physical examination revealed a body temperature of 38.1 °C, respiratory rate of 40/min, heart rate of 90 beats/min, normal color of mucous membranes, dehydration degree: 2%, Capillary Refill Time: 1 s, and normal size of submandibular lymph nodes. An orthopedic examination revealedpain during flexion and extension movements, abnormal mobility, local sensitivity, crepitation, and loss of function in the elbow. Laboratory examinations revealed no abnormality and all values were within physiological limits. Two-way X-ray images of the left forelimb were taken in anteroposterior (A/P) and mediolateral (M/L) positions, revealing a proximal fracture in the left olecranon (Figure 1). The operation was planned 24 hours after the evaluation of the radiographs. The patient was not given food until 12 hours preoperatively and water until 6 hours preoperatively. The operation site was prepared for surgery, including the upper and lower joints of the bone where the fracture was located.

Following disinfection of the area with routine asepsis-antisepsis methods, induction was performed with 10 mcg/kg medetomidine hydrochloride (Domitor, 1 mg/1 mL, Zoetis®, İstanbul, Türkiye) intravenously (IV) and

propofol (propofol, 10 mg/mL, Polifarma®, Tekirdağ, Türkiye) at 2 mg/kg IV. The patient was oro-tracheally intubated and then maintained with ısoflurane (2% Isoflurane® USP, USA) (Kumandas et al., 2019). Sterile drapes surrounded the operation site. A skin incision was made over the lateral elbow and the fracture fragments were exposed. Two Kirschner wires (K-wires) were retrogradely inserted into the ulna. One K-wire was applied transversally distal to the fracture fragment. The 2 K-wires which were placed to the olecranon were bent to the lateral and medial to the point where they would come together with the ends of the transversally inserted pin. The ends of the wires were attached with epoxy material (Kurt & Sen, 2022). The incision site was closed with simple interrupted sutures using 2-0 monofilament atraumatic (GMD®, İstanbul/Türkiye) suture material (Figure 2).

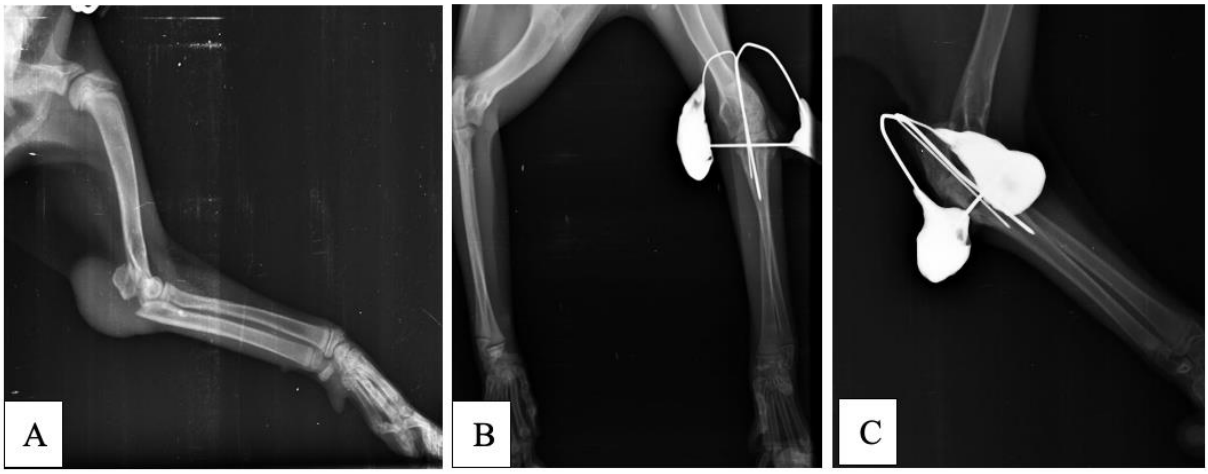

Figure 1. Preoperative M/L (A), postoperative A/P (B), and M/L (C) X-ray images of the patient.

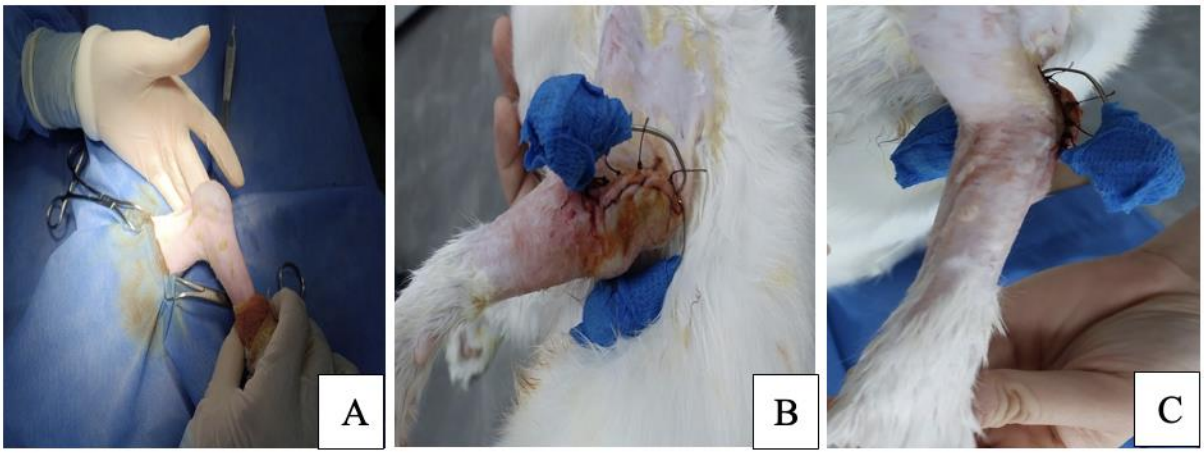

Figure 2. Preoperative view of the surgical site (A); (B) and (C) postoperative view of the implant material.

After the operation, the patient received cefazolin Na (20 mg/kg, 12h, Iespor®, İ.E. Ulagay, İstanbul, Türkiye) intramuscularly (IM) for 7 days, meloxicam (2 mg/kg, 24h, Bavet Meloxicam®, 5 mg/ml, İstanbul, Türkiye) subcutaneously (SC) for 3 days, and sucralfate (1 mg/kg, 12h, Antepsin®, Bilim, Kocaeli, Türkiye) orally for 3 days. To prevent pin site infections, the patient owner was advised to clean the pin site with 10% povidone-iodine (Biokadin®, Adeka, İstanbul, Türkiye) at least twice a day and to wear an Elizabethan collar until the implants were removed. Functional use of the limb with full weight-bearing was

observed immediately after recovery from anesthesia. On day 62, the pins were easily removed under sedation (Figure 3). After the pins were removed, the patient continued to use his extremity comfortably, and X-ray showed complete union of the fracture line.

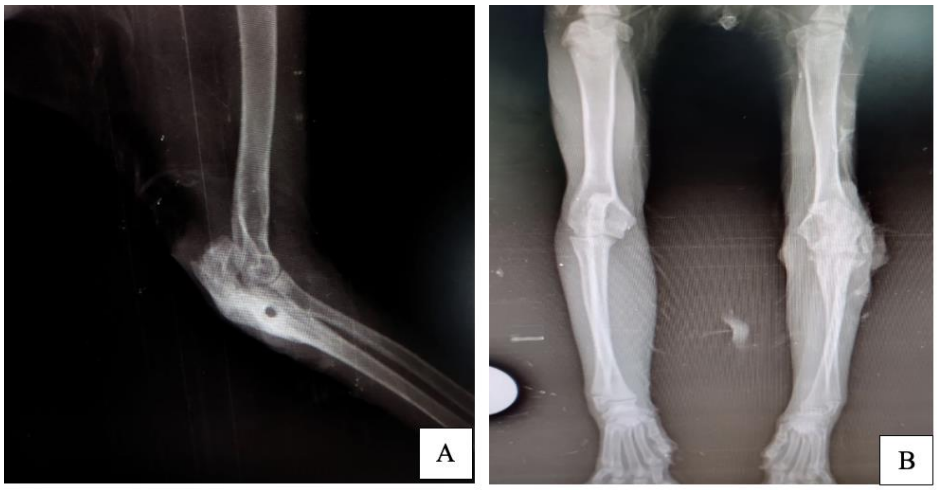

Figure 3. Postoperative 62nd day, fixator removed callus formation can be seen in M/L (A) and A/P (B) X-ray images.

Discussion

Fractures of the antebrachium are common in small animals. In contrast, olecranon fractures are uncommon (Houlton & Dunning, 2005). Because most olecranon fractures are articular, anatomical reduction and internal fixation are usually required to counteract the tensile forces exerted by the triceps brachii muscle group, promote primary bone healing, mitigate the development of posttraumatic osteoarthritis, and optimize the probability of returning to pre-fracture limb function (Fox, 2012). Different techniques are used for the treatment of olecranon fractures in small animals. Tension band wiring and plate osteosynthesis are two techniques currently recommended by the *Arbeitsgemeinschaft für Osteosynthesefragen* (AO) Vet group for olecranon fracture repair (Paul et al., 2021). Proximal ulnar fractures may be articular or nonarticular. Nonarticular ulnar fractures in the proximal elbow joint are usually avulsion fractures of the olecranon. Because of the traction of the triceps brachii muscle group, tensile forces must be resisted when repairing olecranon fractures. In this state there is anatomical reduction and internal fixation are usually required to counteract the tensile forces exerted by the triceps brachii muscle group, promote primary bone healing, reduce the development of posttraumatic osteoarthritis, and optimize the likelihood of return to function of the pre-fracture limb (Johnston & Tobias, 2018; Şen & Kaya, 2018; Şen & Sağlam, 2021).

In this case, 2 K-wires were inserted retrograde through the ulna, and another K-wire was inserted transversally to the distal fragment to counteract the tensile force of the triceps brachii muscle, and we combined this K-wire with the two previously applied K-wires with epoxy material. Stabilization of the olecranon osteotomy is usually achieved with 2 pins or K-wires and a tension band wire (Piermattei et al., 2006). In this case report, parallel to the other reports the epoxy-pin ESF application counteracted the tensile forces exerted on the fracture fragment by muscles, ligaments, and tendons and converted them into compressive forces. Compression of the fracture fragments reduces the width of the

fracture gap and promotes primary bone healing by supporting inter-fragment stability (Piermattei et al., 2006; Şen, 2018; Karslı, 2022).

Olecranon fracture repair has a high complication rate of approximately 37% in dogs, including osteomyelitis, loss of reduction, and migration of K-wires (Paul et al., 2021). Since we applied an ESF in our case, there was no pin migration or loss of reduction that would cause early removal of the fixator. A recent report compared the standard tension band technique with circular external fixation for stabilization of olecranon osteotomies in dogs. Although the reduction accuracy and yield load were similar, the circular fixator provided greater initial stiffness and resisted a higher load. Possible advantages of the ESF method include the fact that fixation eliminates the need for external coaptation, provides unobstructed access for wound cleaning when necessary, and implants can be easily removed when no longer needed (Verpaalen et al., 2020). In this case report, weight-bearing started early, wound cleaning was easy, and the fixator was easily removed when fracture healing was completed.

With the ESF method we applied, the patient started to use his leg shortly after theoperation, there was no need for bandage application after the tension band application, thus, muscle atrophy was not formed and complications such as bandage cuts caused by the bandage were not formed. In addition, the implants were easily removed without requiring invasive surgery. Tissue trauma during implant removal was also prevented. External fixation is an effective method to stabilize many fractures in veterinary orthopedics (Bakici et al., 2019; Gülaydın et al., 2019; Şen, 2020). Although careful attention to fixator selection and application principles is the best way to minimize complications, they can still occur. The vast majority of complications are superficial pin tract infection and subsequent implant failure. However, prompt recognition of these complications and appropriate treatment will minimize their impact and ensure a successful outcome of fracture healing and normal limb function (Beever et al., 2018). In our case report, no complication such as pin site infection, and pin site cleaning was performed regularly to prevent this situation.

Conclusion

After the operation, the patient was able to use the extremity. Thus, no muscle atrophy was observed. At the same time, the materials used were easily removed after the fracture healing. Tie-in type external fixation has not been previously applied to feline proximal olecranon fractures. In this case presentation, a successful fracture union was obtained by utilizing the advantages of an ESF and avoiding the complications of internal fixator applications. Thus, an alternative method for intervention of olecranon fractures was presented.

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Ethical Statement

This study does not present any ethical concerns.

Author Contributions

Investigation: B.K.K.; E.S.A, A.A.; Material and Methodology: B.K.K.; E.S.A.; Supervision: B.K.K.; A.A.; E.A.; O.B.; Visualization: B.K.K.; E.S.A., A.A; Writing-Original Draft: B.K.K.; E.S.A.; A.A.; Writing review & Editing: B.K.K.; E.S.A.; A.A; O.B.

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Starch Based Edible Films and Coatings

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Abstract
While packaging is effective in reducing food losses, the increased use of petroleum-based packaging materials negatively impacts the environment. Edible films and coatings are considered a significant alternative in addressing this issue. Starch, due to its abundant presence in nature, biodegradable nature, and cost-effectiveness, is a widely studied biopolymer. However, its mechanical properties and sensitivity to moisture limit its use as a food packaging material. This article compiles the characteristics, production, and recent studies on starch-based edible films and coatings.
Keywords: Edible film and coating, food packaging, starch.

Nişasta Bazlı Yenilebilir Film ve Kaplamalar

Öz
Ambalaj, oluşacak gıda kayıplarının azaltılmasında etkili olurken artan petrol bazlı ambalaj materyalleri çevreyi olumsuz yönde etkilemektedir. Yenilebilir film ve kaplamalar mevcut sorunun çözümünde önemli bir alternatif olarak öngörülmektedir. Nişastanın doğada bol bulunması, biyobozunur özelliği ve ekonomik olması nişastayı en çok çalışılan biyopolimer kılmaktadır. Buna rağmen sahip olduğu mekanik özellikler ve nem duyarlılığı gıda ambalajı olarak kullanımını sınırlamaktadır. Bu makalede, nişasta bazlı yenilebilir film ve kaplamaların özellikleri, üretimleri ve son yıllarda yapılan çalışmalar derlenmiştir.
Anahtar kelimeler: Yenilebilir film ve kaplama, gıda ambalajı, nişasta.

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Introduction

Packaging is a communication tool that prevents biological, microbiological, physical, and chemical deterioration in foods, thereby extending the shelf life of foods, facilitating their use, and providing consumers with essential information about the food (Üçüncü, 2011). In the production of food packaging, plastic, a petroleum-based polymer, is widely used due to its economic benefits and ease of use (Matthews et al., 2021). Approximately 36% of plastic, widely used in various industries, is employed as packaging material, with 86% of it ending up as uncontrolled waste (UNEP, 2022). Environmental pollution caused by the slow decomposition of plastic in nature has been increasing (Dissanayake et al., 2022). Additionally, microplastics resulting from plastic breakdown enter the food chain, leading to various health issues (Zhu et al., 2024). Consequently, there is an increasing interest in edible and biodegradable environmentally friendly materials in recent years with research focusing in that direction (Mohamed et al., 2020).

Starch, a polysaccharide-type polymer, is abundantly found in nature. It is also cost-effective, tasteless, odorless, and biologically safe. Due to these characteristics, starch plays a promising role in replacing plastic (Almasi et al., 2010). This study takes over the chemical structure and properties of starch, as well as the characteristics of edible films derived from starch.

Chemical Structure and Properties of Starch

Starch is a carbohydrate synthesized by plants through photosynthesis to store excess glucose. It is a homopolysaccharide composed of granules. Starch granules, commonly found in seeds, roots, and tubers of plants, exhibit various shapes and sizes, such as spherical, polygonal, oval, or disk-like (Shannon et al., 2009). These granules consist of two biopolymers, namely amylose and amylopectin, arranged in successive order. The glucose units in the amylose polymer are mostly linear with α-(1-4) linkages and minimal branching, whereas in the amylopectin polymer, glucose units are branched network with α-(1-4) and α-(1-6) linkages (Bello-Perez & Agama-Acevedo, 2018). The proportions of these polymers within starch granules vary, influencing the gelatinization, thermal processing, retrogradation, and rheological properties of starch (Karakelle et al., 2020). The amounts of amylose and amylopectin obtained from plants vary, and based on the amylose/amylopectin ratio, starch is categorized as normal, waxy, or resistant starch (Clerici et al., 2019) (Table 1).

Table 1. Amylose and amylopectin contents of starches

Starch	Amylose (%)	Amylopectin (%)
Normal	18-35	70-82
Waxy	<8	92-100
Resistant	≥50	≤50

The detailed process of starch production dates back to around 184 B.C. According to a document written by the Roman Cato, starch was obtained by soaking grains in water for 10 days, followed by pressing and filtering the mixture. The precipitate in the filtrate was then washed, and the resulting sediment was dried in the sun (Shannon et al., 2009). This method sheds light on the traditional extraction method used today. Additionally, the recovery of starch is possible through ultrasonically assisted and supercritical extraction methods. Furthermore, the alkali and acid solutions used in extraction methods contribute to enhancing the purity of the obtained starch (Palacios-Fonseca et al., 2013).

Starch finds applications in various industries such as food, paper, chemistry, and packaging. In the food industry, it is extensively used as a fundamental component or additive in the

production and preservation of products. The involvement of starch in the packaging sector is facilitated by edible films and coatings (Vilpoux & Junior, 2022).

Starch-Based Films

Preparation of Starch Films

The production of starch-based films utilizes the gelatinization property of starch, which involves the swelling of starch granules through water absorption under high temperatures. There are two methods for the gelatinization of starch granules: one involves applying heat in the presence of high humidity, while the other is achieved through extrusion under low humidity conditions. Extrusion is a shaping process conducted under high temperature and pressure. The properties of the films obtained from both processes vary depending factors such as the starch composition, plasticizer used in the film solution, polymer, and other additives (Lumdubwong, 2019).

The extrusion method for obtaining starch-based films is a less preferred technique. In recent years, high hydrostatic pressure has been applied as an alternative to heat treatment. Films prepared using high hydrostatic pressure have shown improved mechanical properties, lower water vapor permeability, and enhanced thermal stability (Kim et al., 2018).

Starch Film Properties

Starch is a non-toxic polymer with the ability to form good films. The properties of these films are influenced by the source of plan tor fruit from which the starch is derived (Pajak et al., 2019). Starch-based films are transparent, odorless, tasteless, and exhibit good oxygen barrier properties (Cheng et al., 2022). However, they have inadequate moisture barrier and weak mechanical properties. These unfavorable characteristics in films made from pure starch limit their use as packaging material (Nordin et al., 2020). To address these limitations, plasticizers need to be used and/or different polymer combinations need to be created (Li et al., 2024). Starch films prepared with plasticizers such as glycerol and sorbitol show an increase in water solubility, water vapor permeability, and elongation percentage, whereas the inclusion of mannitol and sorbitol reduces water vapor permeability (Ballesteros-Martinez et al., 2020; Ma et al., 2023). Moreover, films prepared with a combination of glycerol, thymol, and corn starch exhibit a decrease in water vapor permeability and tensile strength, and an increase in elongation at break. Additionally, thymol added to the film combination imparts ultraviolet (UV) light barrier properties (Nordin et al., 2020).

The addition of plant and fruit extracts as fillers to the prepared films improves their mechanical and physical properties (Table 2). The phenolic compounds found in these extracts provide the films with antioxidant and antimicrobial activity (Ali et al., 2019; Menzel et al., 2019). Furthermore, anthocyanins present in some plant extracts add smart packaging features to the films by causing color changes at different pH ranges. This enables the visual assessment of potential quality losses and spoilage in food products (Cheng et al., 2022).

In films created with the addition of various extracts, their properties vary depending on the characteristics of the extract. For example, extracts obtained from plants, fruits, and microalgae confer antioxidant properties to the films. Essential oils, owing to their hydrophobic nature, impart antibacterial properties by disrupting the bacterial cell membrane (Carissimi et al., 2018; Shen et al., 2022).

Table 2. Effect of some extracts and oils used on starch-based films

Type of extract or essential oil	Components forming the films	Effects	References
Basil leaf extract	Polyvinyl alcohol (PVA) + starch + %15 basil extract	Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* Low tensile strength	Varghese et al., 2023
Portulaca oleracea extract (POE)	Chitosan + wheat and pea starch (1:1) + glycerol + POE	Increased water vapor permeability Low tensile strength, high elongation at break High antioxidant property	Fan et al., 2023
Rosemary leaf extract	Cassava starch + glycerol + rosemary extract	UV light barrier Antioxidant properties	Piñeros-Hernandez et al., 2017
Rose petal extract	Buckwheat starch + citric acid + rose petal extract	Antioxidant properties UV light barrier Low tensile strength, high elongation at break pH sensitivity	Thakur et al., 2023
Pomegranate peel extract	Taro starch + casein + glycerol + pomegranate peel extract	Antimicrobial activity against *E.coli* and *S.aureus* High thermal stability	More et al., 2022
Honey bee extracts	Starch + glycerol + honey bee extract	Low tensile strength Better structure and mechanical properties in bee bread and propolis extracts	Pajak et al., 2022
Red cabbage extract	Red cabbage extract (RCE) + sweet whey (SW) + glycerol + starch	T2 (64,18% RCE+4,36% SW), T7(50% RCE + 0% SW), T10 (50% RCE +15 SW) films showed higher mechanical and antioxidant; lower humidity and solubility properties	Sanches et al., 2021
Oregano essential oil (OEO)	Dioscorea zingiberensis starch + glycerol + Oregano essential oil	UV light barrier Antioxidant properties 3% oil concentration antimicrobial effect against *Bacillus subtilis, E.coli, S.aureus*	Shen et al., 2022
Orange essential oil	Corn starch + glycerol + orange essential oil	Low tensile strength and elongation at break Antimicrobial activity against *Listeria monocytogenes* and *S. aureus* High water vapor permeability	do Evangelho et al., 2019
Cinnamon essential oil	Cassava starch + glycerol + Tween 80 + cinnamon essential oil	Low tensile strength and high elongation at break High O_2 barrier UV light barrier Low water vapor permeability High thermal stability	Zhou et al., 2021

Starch-Based Edible Films in Food Packaging

Edible films and coatings contribute to preserving the physical and chemical properties of foods, preventing potential quality losses, and extending shelf life by inhibiting pathogenic microbial activation. The selection of the polymer, plasticizer, and other additives used in film production should be tailored to the specific characteristics of each food, and an appropriate film solution should be prepared accordingly (Li et al., 2024).

Additionally, mechanical damages on the surface of foods may increase microbial activation. Therefore, packaging should be resistant to mechanical damages, and edible film surfaces should not crack during storage methods such as refrigeration or freezing. Particularly in the packaging of protein-rich red meats, poultry, fish, and dairy products, a barrier property against potential pathogenic microorganisms should be considered (Üçüncü, 2011).

Edible food packaging should possess certain features depending on the type of food it will be applied to. For example, in the packaging of fresh meats, high oxygen permeability is required to maintin the bright red color of the meat, while low water vapor permeability is necessary to prevent water loss (Sezer & Bozkurt, 2021). For frozen meats, packaging with low oxygen and water vapor permeability is preferred to prevent water loss and lipid oxidation. Since fish is rich in unsaturated fatty acids, measures should be taken to prevent deoxidative spoilage. The packaging used for ripened cheeses should have high water vapor permeability to prevent moisture loss, which can lead to, weight and quality losses. For ripened cheeses to maintain the activities of their flora without quality loss, gas barrier permeability is also required. Different types of cheese have varied characteristics, necessitating different packaging conditions. Semi-hard and soft cheeses require low light, water vapor, and gas barrier properties, while shell-less cheeses require non-water vapor-permeable packaging, and fresh cheeses require packaging with low light, water vapor, and gas permeability (Üçüncü, 2011).

In the packaging of fresh fruits and vegetables, providing an appropriate gas and moisture barrier according to the type of food can prevent weight, taste, and odor losses, as well as inhibit browning reactions (Chettri et al., 2023).

Numerous studies have been conducted on the use of starch-based edible films in various food products (Baek et al., 2019; De Moraes et al., 2020; Mehdizadeh et al., 2020; Carrión et al., 2023; Fan et al., 2023; Abera et al., 2024; Bodana et al., 2024; Da Costa et al., 2024)

In a study conducted by Baek et al. (2019), navy bean starch films containing maki fruit extract were examined for antioxidant properties on salmon samples. The linear increase in antioxidant activity was observed with the increased proportion of maki fruit extract in the film content. Additionally, the film, which created a UV light barrier, prevented lipid oxidation in salmon samples.

De Moraes et al. (2020) prepared antimicrobial films treated with pulsed light, including a starch combination of sodium benzoate, citric acid, and sodium benzoate + citric acid. The antimicrobial properties of the film were examined on sliced cheddar cheese inoculated with *Listeria innocua*. Pulsed light application did not show a strong effect on the film structure. While the concentration of sodium benzoate used in the study did not exhibit antimicrobial effects, citric acid demonstrated its impact.

In a study by Mehdizadeh et al. (2020), chitosan-starch film containing pomegranate peel extract and thyme essential oil was applied to beef and monitored for 21 days at 4 °C. The film showed

antimicrobial effects against *Listeria monocytogenes* and prevented lipid oxidation in beef, thereby extending shelf life.

Carrión et al. (2023) applied sodium alginate, nisin, and taro starch-based films to chicken meat inoculated with *L. monocytogenes* and investigated their antimicrobial effects during refrigerated storage. The prepared film exhibited antimicrobial effects against *L. monocytogenes*, extending the shelf life by 15 days compared to the control sample.

In a study by Fan et al. (2023), the potential application of purslane extract, chitosan, and wheat + pea starch-based films in chilled pork was examined. Pork slices of 8x8 cm were stored at 4 ˚C for 16 days. The antioxidant properties imparted to the film due to the extract used in the film solution prevented lipid oxidation in chilled meat, thereby preventing spoilage and extending shelf life.

Abera et al. (2024) created a film combination using starch, chitosan, and glycerol. The solution formed was applied to apples, mangoes, and strawberries. The resulting films extended the shelf life, with apples and mangoes showing an increased shelf life of 28 days and strawberries exceeding 21 days with minimal weight loss.

Bodana et al. (2024) produced jackfruit starch-based films containing pomegranate peel extract to extend the shelf life of white grapes. It was observed that the firmness of white grapes was maintained during storage at room temperature for up to 8 days, with reduced weight loss and acceptable color preservation.

Da Costa et al. (2024) aimed to extend the shelf life of fresh pears by producing k-carrageenan-starch films containing copper oxide particles. The added copper oxide particles provided UV barrier properties to the film, and no quality loss was observed during a 30-day storage period.

Conclusion

In conclusion, the abundance of starch in nature and its ability to form effective films offer promising prospects for it to replace traditional packaging materials. However, its susceptibility to moisture limits its applicability. Nevertheless, films with better physical and mechanical properties have been observed by incorporating plasticizers, polymers, and other additives into the created combinations. The addition of extracts, essential oils, and other bioactive compounds to starch-based films provides antioxidant and antimicrobial properties, turning them into active and intelligent packaging. These films effectively prevent quality losses and microbial activity, leading to extended shelf life in applied food products. Further research is needed to explore the extrusion process in starch-based film production, and combinations of films created from starch obtained from various sources are expected to have a significant impact on the literature.

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Ethical Statement

This study does not present any ethical concerns.

Author Contributions

Investigation: K.Y.B.; Supervision: H.Ç.; Visualization: K.Y.B.; Writing-Original Draft: K.Y.B. Writing- review & Editing: K.Y.B. & H.Ç.

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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