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Volume: 30, Issue: 1, Year: 2024

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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 1, Year: 2024

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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 1, Year: 2024

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 1, Year: 2024

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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Editorial

EDITORIAL: Time to Treat the Climate and Nature Crisis as One Indivisible Global Health Emergency

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Over 200 health journals call on the United Nations, political leaders, and health professionals to recognise that climate change and biodiversity loss are one indivisible crisis and must be tackled together to preserve health and avoid catastrophe. This overall environmental crisis is now so severe as to be a global health emergency.

The world is currently responding to the climate crisis and the nature crisis as if they were separate challenges. This is a dangerous mistake. The 28th Conference of the Parties (COP) on climate change is about to be held in Dubai while the 16th COP on biodiversity is due to be held in Turkey in 2024. The research communities that provide the evidence for the two COPs are unfortunately largely separate, but they were brought together for a workshop in 2020 when they concluded that: "Only by considering climate and biodiversity as parts of the same complex problem…can solutions be developed that avoid maladaptation and maximize the beneficial outcomes" [1].

As the health world has recognised with the development of the concept of planetary health, the natural world is made up of one overall interdependent system. Damage to one subsystem can create feedback that damages another-for example, drought, wildfires, floods and the other effects of rising global temperatures destroy plant life, and lead to soil erosion and so inhibit carbon storage, which means more global warming [2]. Climate change is set to overtake deforestation and other land-use change as the primary driver of nature loss [3].

Nature has a remarkable power to restore. For example, deforested land can revert to forest through natural regeneration, and marine phytoplankton, which act as natural carbon stores, turn over one billion tonnes of photosynthesising biomass every eight days [4]. Indigenous land and sea management has a particularly important role to play in regeneration and continuing care [5].

Restoring one subsystem can help another-for example, replenishing soil could help remove greenhouse gases from the atmosphere on a vast scale [6]. But actions that may benefit one subsystem can harm another-for example, planting forests with one type of tree can remove carbon dioxide from the air but can damage the biodiversity that is fundamental to healthy ecosystems [7].

The Impacts on Health

Human health is damaged directly by both the climate crisis, as the journals have described in previous editorials [8,9], and by the nature crisis [10]. This indivisible planetary crisis will have major effects on health as a result of the disruption of social and economic systems-shortages of land, shelter, food, and water, exacerbating poverty, which in turn will lead to mass migration and conflict. Rising temperatures, extreme weather events, air pollution, and the spread of infectious diseases are some of the major health threats exacerbated by climate change [11]. "Without nature, we have nothing," was UN Secretary-General António Guterres's blunt summary at the biodiversity COP in Montreal last year [12]. Even if we could keep global warming below an increase of 1.5ºC over pre-industrial levels, we could still cause catastrophic harm to health by destroying nature.

Access to clean water is fundamental to human health, and yet pollution has damaged water quality, causing a rise in water-borne diseases [13]. Contamination of water on land can also have far-reaching effects on distant ecosystems when that water runs off into the ocean [14]. Good nutrition is underpinned by diversity in the variety of foods, but there has been a striking loss of genetic diversity in the food system. Globally, about a fifth of people rely on wild species for food and their livelihoods [15]. Declines in wildlife are a major challenge for these populations, particularly in low- and middle-income countries. Fish provide more than half of dietary protein in many African, South Asian and small island nations, but ocean acidification has reduced the quality and quantity of seafood [16].

Changes in land use have forced tens of thousands of species into closer contact, increasing the exchange of pathogens and the emergence of new diseases and pandemics [17]. People losing contact with the natural environment and the declining biodiversity have both been linked to increases in noncommunicable, autoimmune, and inflammatory diseases and metabolic, allergic and neuropsychiatric disorders [10,18]. For Indigenous people, caring for and connecting with nature is especially important for their health [19]. Nature has also been an important source of medicines, and thus reduced diversity also constrains the discovery of new medicines.

Communities are healthier if they have access to high-quality green spaces that help filter air pollution, reduce air and ground temperatures, and provide opportunities for physical activity [20]. Connection with nature reduces stress, loneliness and depression while promoting social interaction [21]. These benefits are threatened by the continuing rise in urbanisation [22].

Finally, the health impacts of climate change and biodiversity loss will be experienced unequally between and within countries, with the most vulnerable communities often bearing the highest burden [10]. Linked to this, inequality is also arguably fuelling these environmental crises. Environmental challenges and social/health inequities are challenges that share drivers and there are potential co-benefits of addressing them [10].

A Global Health Emergency

In December 2022 the biodiversity COP agreed on the effective conservation and management of at least 30% percent of the world's land, coastal areas, and oceans by 2030 [23]. Industrialised countries agreed to mobilise $30 billion per year to support developing nations to do so [23]. These agreements echo promises made at climate COPs.

Yet many commitments made at COPs have not been met. This has allowed ecosystems to be pushed further to the brink, greatly increasing the risk of arriving at 'tipping points', abrupt breakdowns in the functioning of nature [2,24]. If these events were to occur, the impacts on health would be globally catastrophic.

This risk, combined with the severe impacts on health already occurring, means that the World Health Organization should declare the indivisible climate and nature crisis as a global health emergency. The three pre-conditions for WHO to declare a situation to be a Public Health Emergency of International Concern [25] are that it: 1) is serious, sudden, unusual or unexpected; 2) carries implications for public health beyond the affected State's national border; and 3) may require immediate international action. Climate change would appear to fulfil all of those conditions. While the accelerating climate change and loss of biodiversity are not sudden or unexpected, they are certainly serious and unusual. Hence we call for WHO to make this declaration before or at the Seventy-seventh World Health Assembly in May 2024.

Tackling this emergency requires the COP processes to be harmonised. As a first step, the respective conventions must push for better integration of national climate plans with biodiversity equivalents [3]. As the 2020 workshop that brought climate and nature scientists together concluded, "Critical leverage points include exploring alternative visions of good quality of life, rethinking consumption and waste, shifting values related to the human-nature relationship, reducing inequalities, and promoting education and learning" [1]. All of these would benefit health.

Health professionals must be powerful advocates for both restoring biodiversity and tackling climate change for the good of health. Political leaders must recognise both the severe threats to health from the planetary crisis as well

as the benefits that can flow to health from tackling the crisis [26]. But first, we must recognise this crisis for what it is: a global health emergency.

This Comment is being published simultaneously in multiple journals. For the full list of journals see: https://www.bmj.com/content/full-list-authors-and-signatories-climate-nature-emergency-editorial-october-2023

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RESEARCH ARTICLE

Beclin-1 Improves the Cognitive Function of Mice with Alzheimer's Disease

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Abstract

We aimed to investigate the improvement effect of Beclin-1 on the cognitive function of mice with Alzheimer's disease (AD). Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) were isolated from neonatal umbilical cord tissues. The cells stably overexpressing Beclin-1 were constructed, and those of passages 5, 10, and 15 were used. Forty-eight AD mice were randomly divided into AD, P5 hUC-MSCs, P15 hUC-MSCs (P15MSCs) and OE Beclin 1-P15 hUC-MSCs (OE Bec-P15MSCs) groups (n=12). Overexpressing Beclin-1 significantly enhanced the proliferation and migration of MSCs of different passages, and reduced G0/G1 arrest. The OE Bec-P15MSCs group had the longest total distance, the shortest time in forced swimming test, and the largest total food consumption in novelty suppressed feeding test. Transplanting hUC-MSCs and overexpressing Beclin-1 significantly reduced Aβ deposition. The expressions of P-tau (Ser396), P-tau (Ser231) and P-tau (Ser235) were significantly inhibited by hUC-MSCs transplantation and Beclin-1 overexpression, especially in the OE Bec-P15MSCs group. Double positive staining of EdU^+/DCX^+ cells, $EdU^+/NeuN^+$ cells and $EdU^+/Nestin^+$ cells significantly increased in the OE Bec-P15MSCs group compared to those in other groups ($P<0.05$). The activation degrees of astrocytes and microglia were lowest and the superoxide dismutase activity was highest in the OE Bec-P15MSCs group ($P<0.05$). The protein expression of nuclear factor E2-related factor 2 (Nrf2) in NAD(P)H quinone oxidoreductase 1 and superoxide dismutase 1 in brain tissues significantly rose, while that of Keap-1 was down-regulated in the OE Bec-P15MSCs group ($P<0.05$). Beclin-1 can partly restore the viability of hUC-MSCs by activating the Nrf2 signaling pathway, thereby enhancing the therapeutic effect of transplantation on AD mice.

Keywords: Aging, Alzheimer's disease, Beclin-1, Mesenchymal stem cell

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder clinically manifested as global dementia characterized by memory impairment, aphasia, apraxia, agnosia, impairment of visuospatial skills, executive dysfunction, and personality and behavioral changes [1]. Amyloid-β (Aβ) deposition and neurofibrillary tangles induced by hyperphosphorylated tau are major pathological hallmarks of AD, which can lead to progressive neuronal loss and memory loss [2]. Recently, embryonic stem cells, mesenchymal stem cells (MSCs), brain-derived neural stem cells and induced pluripotent stem cells have been most commonly used in AD-related studies [3]. MSCs can be extracted from pluripotent stem cells, so they are more easily accessible and abundant than other types of stem cells [4]. Besides, the autologous source of MSCs overcomes the ethical issues related to embryonic stem cells [5].

MSCs are a kind of adult stem cells with self-replication ability and multi-directional differentiation potential, which can be induced to differentiate into a variety of tissue cells under specific circumstances. The immunomodulatory and neuroprotective activities of MSCs have been well-documented [6]. The mice treated with bone marrow-derived MSCs undergo brain changes, including increased synaptic density, decreased ratio of M1/M2 type activated microglia, and elevated levels of anti-inflammatory and neuroprotective cytokines such as

CXCL5, MCP-1, β-NGF, TIMP-1, VEGF-A, TGF-β and IL-10 [7]. MSC-based replacement therapy has become a potential treatment strategy for AD [8]. Transplantation of human umbilical cord-derived MSCs (hUC-MSCs) can improve the cognitive function of AD animal models by reducing Aβ deposition, protecting neuronal integrity and promoting neurogenesis [9]. However, stem cells, like other cells, will gradually develop a senescent phenotype, leading to a gradual decline in cell function. With the increase of passage times, hUC-MSCs cultured *in vitro* are prone to decreased proliferative activity and differentiation capacity, seriously affecting the therapeutic effect [10,11]. Therefore, it is urgently needed to explore new strategies for delaying the aging process of hUC-MSCs and increasing their viability to ensure post-transplantation efficacy.

Beclin-1 is one of the key parts of class III phosphatidylinositol 3-kinase complex, and its abnormal level is closely associated with cancers and neurodegenerative diseases [12,13]. Additionally, the overexpression of Beclin-1 has been reported to protect MSCs from apoptosis and inflammation-induced membrane damage [14]. However, whether Beclin-1 can rejuvenate aged hUC-MSCs and enhance their neuroprotective effects in the chronic pathological state of AD remains to be further studied.

Material and Methods

A- *In vitro* Experiments

Isolation and Culture of hUC-MSCs and Construction of Cell Lines

Under aseptic conditions, the umbilical cord tissues of healthy full-term neonates born by cesarean section were harvested, the umbilical arteries and veins were carefully removed, and Wharton's jelly was separated, cut into small pieces (about 1 mm) and inoculated into DMEM/F12 complete medium (Gibco, USA), followed by culture in a 5% CO_2 incubator at 37°C. Upon reaching 90% confluency, the cells were passaged at 1:3, and those of passages 5, 10, and 15 (P5, P10, and P15) were used for further experiments [15]. Then the virus suspension containing pEGFP-N1-GFP-Beclin-1 was added to the complete medium, and after incubation at 37°C for 24 h, the medium containing virus particles was discarded and replaced with fresh complete medium. 48 h later, the successful construction of cell lines overexpressing Beclin-1 was confirmed by Western blotting.

Cell Counting Kit-8 (CCK-8) Assay

The viability of hUC-MSCs was detected by CCK-8 assay according to the kit's instructions (Suzhou Ribo Life Science Co., Ltd., China) [16]. Specifically, the cells were inoculated into a 96-well plate at a density of 1×10^5 cells/well, and cultured at 37°C for 12 h, 24 h, and 36 h. Then, 10 μL of CCK-8 solution was added into each well, and after incubation for 30 min, the optical density (OD) value was measured at 456 nm. Finally, the survival rate was calculated: survival rate = $[OD_{treated\ cells}/OD_{control\ cells}] \times 100\%$. The assay was independently repeated three times.

EdU Proliferation Assay

The proliferation ability of hUC-MSCs was detected by EdU proliferation assay [17]. The cells in the logarithmic growth phase were inoculated into a 96-well plate at a density of 4×10^5 cells/well and incubated overnight. 100 μL of EdU solution diluted with medium at 1:1000 was added into each well for 2 h of incubation. After washing three times with PBS, the cells were added with Apollo staining solution, incubated for 30 min, and washed three times with PBS, followed by DAPI staining and methanol fixation. Finally, at least 8 randomly-selected fields were photographed under a fluorescence microscope (200×; Olympus, Japan).

Wound Healing Assay

The migration capacity of hUC-MSCs was evaluated by wound healing assay and Transwell assay [16]. The cells were inoculated into a 24-well plate and starved for 12 h after a cell monolayer was formed. The monolayer was scratched with a 10 μL pipette tip to create a wound, and the floating cell debris was removed by PBS. Finally, the cells were observed and photographed under a light microscope (Olympus, Japan) at 0 h and 24 h.

Transwell Assay

Specifically, 2.5×10^4 cells were prepared into cell suspension with serum-free medium and dropwise added to the upper Transwell chamber, while complete medium containing 10% fetal bovine serum (Gibco, USA) was added to the lower chamber. After 48 h of incubation, the cells were fixed with paraformaldehyde solution for 20 min and stained with crystal violet for 20 min. Then the cells in the upper Transwell chamber were removed with cotton swabs, and the number of cells passing through the membrane in the lower chamber was observed and counted under a microscope.

β-Galactosidase Activity Staining

The number of β-gal$^+$ cells in hUC-MSCs was observed by β-galactosidase activity staining [18]. The cells were inoculated into a 6-well plate. After complete adherence, the cells were washed twice with PBS, added with 1 mL of staining fixative, and incubated at room temperature for 20 min. After washing twice with PBS, the cells were added with 1 mL of SA-β-gal staining solution in each well, and incubated at 37°C overnight. Finally, they were observed and photographed under a light microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and IL-13 in hUC-MSCs were detected using ELISA according to the kits' instructions (Thermo Fisher Scientific, USA). To be specific, 100 μL of cell culture supernatant was added to the plate, incubated and washed, and then the corresponding antibodies (1:100) were added (Cell Signaling Technology, USA). After washing, 100 μL of horseradish peroxidase-labeled streptavidin (1:100) was added to each well. After washing, 100 μL of chromogenic substrate TMB was added to each well for incubation away from light for 10 min, and the reaction was terminated by 100 μL of stop buffer in each well. Finally, the OD values at 450 nm and 570 nm were measured with a microplate reader.

Detection of Oxidative Stress-related Factors

The reactive oxygen species (ROS) content in hUC-MSCs was measured using ROS assay kit (Beyotime Biotechnology, Shanghai, China). The cells were inoculated into a 12-well plate and cultured until reaching 85% confluency before modeling. After washing with PBS, 1 mL of 10 μM DCFH-DA working solution was added to each well, and the cells were incubated in an incubator at 37°C away from light for 30 min. Then the cells were washed twice with serum-free medium, and rapidly observed and photographed under a fluorescence microscope. After ultrasonication, hUC-MSCs were collected, and 300 μL of MDA working solution (Solarbio Biotech, Wuhan, China) and 100 μL of samples were mixed, heat-preserved in a 100°C water bath for 60 min, and cooled in an ice bath, followed by centrifugation at 10000×g and room temperature for 10 min. Finally, 200 μL of supernatant was added to a 96-well plate, and the OD values were measured at 532 nm and 600 nm.

B- *In vivo* Experiments

Ethical Approval

The human experiment regarding the collection of umbilical cord tissues has been approved by the ethics committee of Xuzhou First People's Hospital on January 4th, 2021 (Approval No. XFPH202101003). All animal experiments have been approved by the animal experiments ethics committee of Xuzhou First People's Hospital on March 8th, 2021 (Approval No. XFPH-dong202103004), and great efforts have been made to minimize the animals' suffering.

Animal Grouping and Treatment

To further verify the improvement effect of Beclin-1 on the cognitive function of AD mice, forty-eight 6-month-old APP/PS1 transgenic mice (AD model mice) purchased from Jackson Medical Technology (Shanghai) Co., Ltd. were randomly divided into AD group, P5 hUC-MSCs (P5MSCs) group, P15 hUC-MSCs (P15MSCs) group and OE Beclin 1-P15 hUC-MSCs (OE Bec-P15MSCs) group (n=12). For the AD group, the same volume of normal saline was intravenously injected. For P5MSCs, P15MSCs and OE Bec-P15MSCs groups, corresponding cells overexpressing Beclin-1 were injected intravenously once daily, respectively, with approximately 1×10^6 cells per injection for 3 d.

Behavioral Tests

Then the cognitive function, anxiety and depression behavior of mice were assessed by Morris water maze (MWM), new object recognition (NOR), open field testing (OFT), forced swimming test (FST), tail suspended test (TST) and sucrose preference test (SPT) [19].

Nissl Staining

Following the behavioral tests, the venous blood was drawn and centrifuged, and the upper serum was collected and stored at -80°C. After the end of the experiment, the mice were anesthetized and sacrificed, and the brain tissues were separated. Finally, the brain tissues were dehydrated, frozen, embedded with OCT and serially sectioned at a thickness of 10 μm with a cryostat. The brain sections were stained with cresol purple to assess neuronal loss and integrity of Nissl bodies. The frozen sections were subjected to gradient heating, placed in cresol purple dye, incubated in an incubator at 56°C for 1 h, washed twice and added with Nissl differentiation solution for 1 min. Finally, the sections were dehydrated, transparentized, and photographed under a microscope [20].

TUNEL Staining

The sections were deparaffinized, hydrated, treated with proteinase K (10 μg/mL) for 15 min, incubated with TUNEL mixture for 60 min, and washed twice with PBS, followed by nuclear staining with 0.1 μg/mL DAPI dye. Finally, the number of TUNEL-positive cells was counted in eight randomly-selected fields per sample under a fluorescence microscope [20].

Immunofluorescence Staining

The sections were placed in citric acid repair buffer, heated by microwave for 6 min, cooled naturally, washed twice with PBS, permeabilized with 0.2% Triton X-100 for 15 min, and washed twice with PBS. After PBS was gently wiped off, the sections were incubated with blocking serum for 1 h, and washed twice with PBS, followed by incubation with the corresponding primary antibodies (Cell Signaling Technology, USA) at 4°C overnight, washing three times with PBS, incubation with fluorescent secondary antibodies (Cell Signaling Technology, USA) at room temperature for 2 h, and washing twice with PBS.

Table 1. *Primer sequences*

Gene	Forward	Reverse
p16	5'-AACACTATGAGGAGCACC-3'	5'-AACGGTAAGCCACGTAGT-3'
p21	5'-CTTTGCCGACCTCGCTCC-3'	5'-CTAACGGTGATTCATGGT-3'
p53	5'-AACTATTAAGGCGACTCG-3'	5'-AGGTTTACGACTATATAG-3'
GAPDH	5'- CAAAT AAACCATCTC GA-3'	5'-AAGAATCGCACC TAGCG-3'

Then the nuclei were stained with DAPI for 15 min, and the sections were washed with PBS, mounted, observed and photographed under a fluorescence microscope [20].

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells or tissues using RNA extraction kit (TaKaRa, Japan). According to the manufacturer's instructions, the total RNA was reversely transcribed into cDNA and amplified using PrimeScript RT Master Mix (TaKaRa, Osaka, Japan) and SYBR Premix Ex Taq Kit (TaKaRa, Osaka, Japan). With GAPDH as an internal reference gene, the relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in *Table 1*.

Western Blotting

Total protein was extracted from cells or tissues using cell total protein extraction kit (Sangon Biotech (Shanghai) Co., Ltd., China). After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beijing Solarbio Biotechnology Co., Ltd., China), the protein was transferred onto a polyvinylidene fluoride membrane, and incubated with primary antibodies against p16, p21, p53, Tau, P-Tau (Ser396), P-Tau (Ser231), nuclear factor E2-related factor 2 (Nrf2), superoxide dismutase 1 (SOD1) and Keap-1 and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, USA). GAPDH and histone 3 were used as internal references for expressions of cytoplasmic and nuclear proteins, respectively.

Statistical Analysis

All assays were independently repeated at least three times, and SPSS20.0 software (IBM Inc., USA) was used for statistical analysis. The measurement data were described by mean ± standard deviation. One-way analysis of variance was used for comparisons among groups, and the LSD-*t* test was conducted for pairwise comparison. $P<0.05$ was considered statistically significant.

Results

Beclin-1 Promoted the Viability, Proliferation and Migration of hUC-MSCs

It was confirmed by Western blotting that hUC-MSC lacked the endogenous expression of Beclin-1, and the protein expression of Beclin-1 was significantly increased after lentivirus infection, indicating the successful construction of cell lines (*Fig. 1-A*). The effects of Beclin-1 on the proliferation and migration of hUC-MSCs of different passages were detected. The results of CCK-8 assay showed that the viability of P10 and P15 MSCs was significantly decreased compared with that of P5 MSCs *(Fig. 1-B)*. In addition, the proportion of P10 and P15 MSCs in G0/G1 phase gradually rose and the proportion of those in S phase declined with the increase of passage times *(Fig. 1-C)*. The results of Transwell migration assay *(Fig. 1-D)*, wound healing assay *(Fig. 1-E)* and EdU proliferation assay *(Fig. 1-F)* revealed that MSCs had gradually weakened migration and proliferation with the increase of passage times. Compared with those of control cells, overexpression of Beclin-1 significantly enhanced the proliferation and migration of hUC-MSCs of different passages, and reduced G0/G1 arrest.

Beclin-1 Inhibited the Senescence of hUC-MSCs

Furthermore, the effects of Beclin-1 on the senescent phenotype of P5, P10, and P15 hUC-MSCs were detected. It was observed by β-galactosidase activity staining that the number of β-gal$^+$ cells in hUC-MSCs gradually rose with the increase of passage times *(Fig. 2-A)*. Meanwhile, immunofluorescence staining also showed that the expression level of Lamin B1, an anti-aging marker, declined gradually with the increase of passage times. Overexpression of Beclin-1 significantly reduced the number of β-gal$^+$ cells and increased the Lamin B1 expression compared with those in untreated cells *(Fig. 2-A)*. As shown by RT-qPCR and Western blotting, the mRNA and protein expressions of p16, p21 and p53 in hUC-MSCs rose with the increase of passage times, while overexpression of Beclin-1 significantly reduced the mRNA and protein expressions of p16, p21, and p53 *(Fig. 2-B,C,D)*. Besides, the results of ELISA showed that overexpression of Beclin-1 significantly inhibited the secretion of IL-1α, IL-1β and TNF-α, and enhanced the release of anti-inflammatory cytokines IL-4, IL-10 and IL-13 in hUC-MSCs *(Fig. 2-E)*. These results suggest that overexpression of Beclin-1 can delay the aging process of hUC-MSCs and rejuvenate aged hUC-MSCs.

Beclin-1 Attenuated Oxidative Stress in Aged hUC-MSCs by Activating Nrf2 Signaling Pathway

Compared with P5 hUC-MSCs, the ROS production significantly rose, while the MDA level and SOD activity

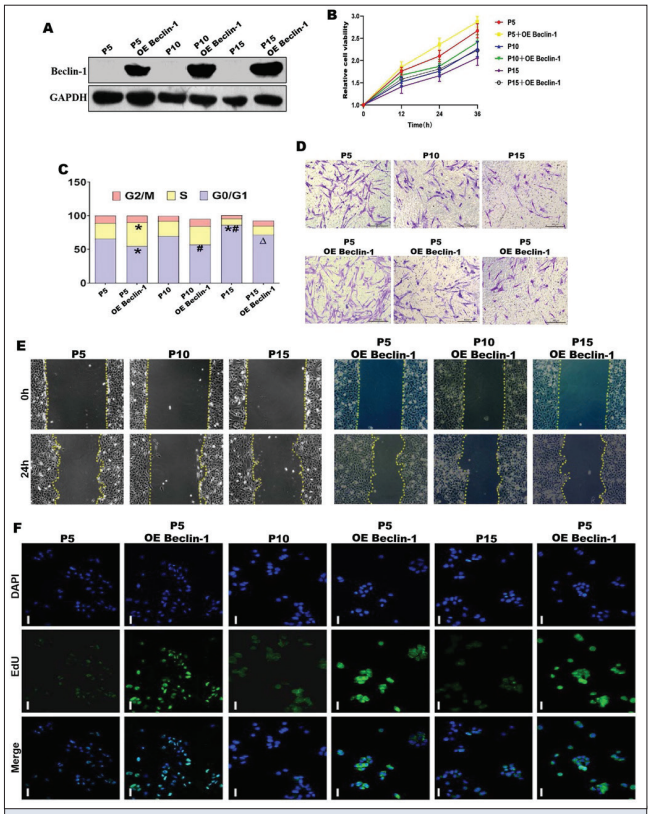

Fig 1. Beclin-1 promoted the viability, proliferation and migration of hUC-MSCs. **A:** Protein expression of Beclin-1detected by Western blotting, **B:** CCK-8 assay for cell viability, **C:** Histogram of cell cycle distribution, **D:** Transwell migration assay, **E:** Wound healing assay, **F:** EdU proliferation assay. The data were described by mean ± standard deviation. *P<0.05 vs. P5 group; #P<0.05 vs. P10 group; ΔP<0.05 vs. P15 group

gradually declined in P10 and P15 hUC-MSCs, which were significantly reversed by overexpression of Beclin-1 *(Fig. 3-A)*. In addition, overexpression of Beclin-1 significantly reduced the expression of Keap-1 in aged hUC-MSCs and enhanced the expressions of Nrf2, NAD(P)H quinone oxidoreductase 1 (NQO1), and SOD1 in the nucleus *(Fig. 3-B,C)*. These results demonstrate that overexpression of Beclin-1 can inhibit oxidative stress in aged hUC-MSCs by activating the Nrf2 signaling pathway.

Overexpression of Beclin-1 Enhanced the Therapeutic Effect of hUC-MSCs in AD Mice

To determine whether overexpression of Beclin-1 can enhance the therapeutic effect of hUC-MSCs, Beclin-1-overexpressing P15 hUC-MSCs were injected into AD mice *via* the tail vein, and the spatial learning and memory function of the mice were evaluated by MWM and NOR tests. As shown in *Fig. 4-A,B,C*, the mice in P5MSCs group had shorter escape latency, more platform-crossing times, longer residence time in the target quadrant and a higher discrimination index than those in AD group (P<0.05). The improvement degree of behaviors was significantly decreased in P15MSCs group, and the behavioral score in OE Bec-P15MSCs group

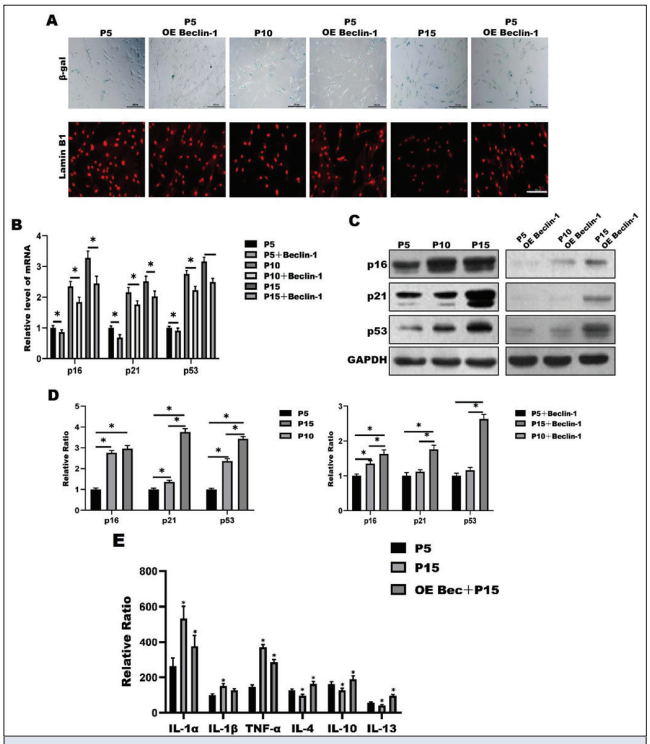

Fig 2. Beclin-1 rejuvenated aged hUC-MSCs. **A:** β-galactosidase activity staining and Lamin B1 immunofluorescence staining, **B:** RT-qPCR. C&D: Western blotting bands and histograms, **E:** Histograms of ELISA results. The data were described by mean ± standard deviation

was significantly better than that in P15MSCs group, indicating that Beclin-1-overexpressing P15 MSCs are superior to P15 MSCs in improving the memory of AD mice. The results of immunofluorescence staining showed that the survival rates of neuronal cells and NeuN$^+$ cells in P15MSCs group were significantly lower than those in P5MSCs group, and they were significantly higher in OE Bec-P15MSCs group than those in P15MSCs group (P<0.05) *(Fig. 4-D)*. The number of TUNEL$^+$ cells in brain tissues was smaller in OE Bec-P15MSCs group than that

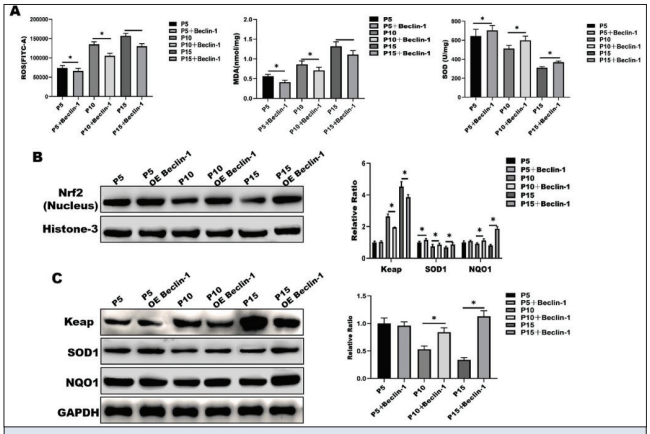

Fig 3. Beclin-1 attenuated oxidative stress in aged hUC-MSCs by activating Nrf2 signaling pathway. **A:** Levels of ROS, MDA and SOD, **B&C:** Western blotting bands and histograms. The data were described by mean ± standard deviation

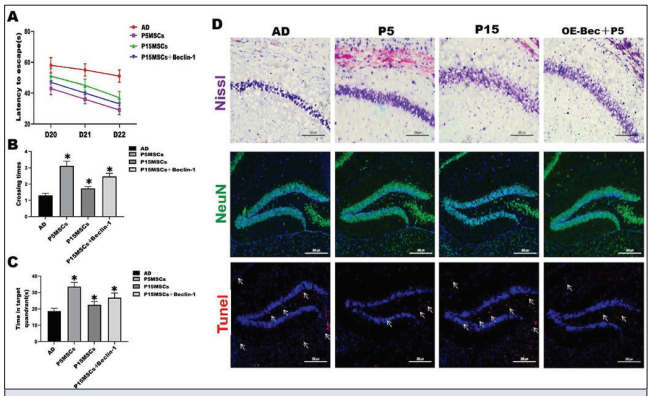

Fig 4. Overexpression of Beclin-1 enhanced the therapeutic effect of hUC-MSCs in AD mice. **A-C:** Histograms of statistical analysis of MWM and NOR test results, **D:** Immunofluorescence staining results of brain tissue sections. The data were described by mean ± standard deviation. *P<0.05 vs. AD group

in P15MSCs group (P<0.05). Beclin-1-overexpressing P15 MSCs promoted the expression of NeuN and reduced the number of TUNEL$^+$ cells in the hippocampus (P<0.05). To sum up, the overexpression of Beclin-1 can improve the therapeutic effect of hUC-MSCs in AD mice.

Beclin-1 Ameliorated Memory Impairment and Depression-like Behavior in AD Mice

OE Bec-P15MSCs group and MSCs groups had more trajectory crossings in the MWM test and longer residence time in the target quadrant than AD group, with the behavioral improvement being the most significant in OE Bec-P15MSCs group. Since most AD patients are usually accompanied by mental symptoms such as anxiety and depression, FST, OFT and NSF tests were also performed to evaluate the mood and behavior of mice. It was found that OE Bec-P15MSCs group had significantly longer

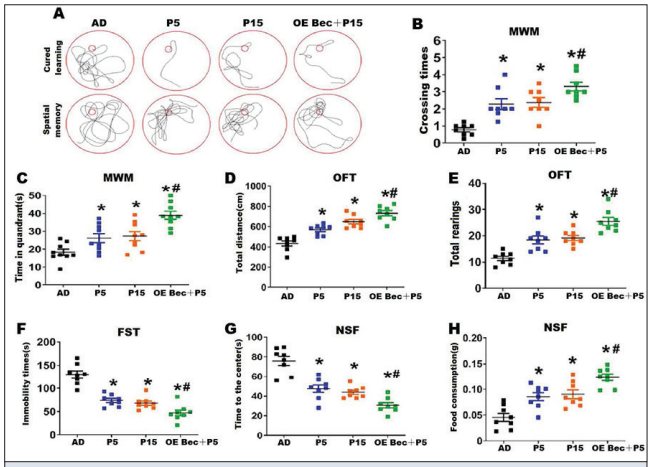

Fig 5. Beclin-1 ameliorated memory impairment and depression-like behavior in AD mice. **A:** MWM test trajectory chart, **B-H:** Histograms of statistical analysis of mouse behavioral test results. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

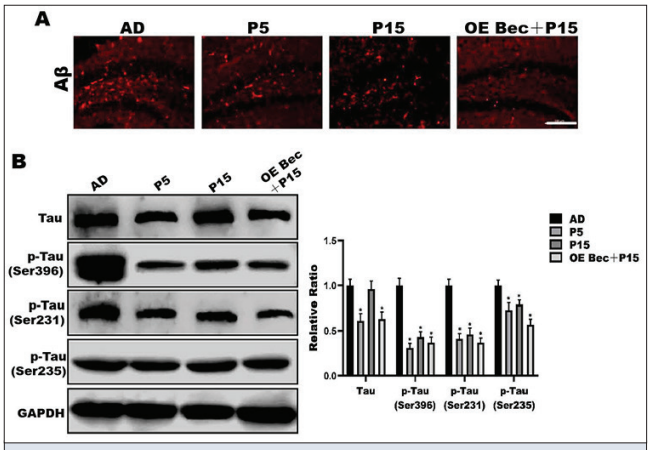

Fig 6. Overexpression of Beclin-1 reduced Aβ deposition and Tau phosphorylation in AD mice. **A:** Immunofluorescence staining of Aβ, **B:** Western blotting bands and statistical analysis histograms. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

swimming time, shorter immobility time and the largest total food consumption than AD and MSCs groups *(Fig. 5)*. It can be inferred that overexpression of Beclin-1 and transplantation of hUC-MSCs can synergistically alleviate memory impairment and depression-like behavior in AD mice.

Overexpression of Beclin-1 Reduced Aβ Deposition and Tau Phosphorylation in AD Mice

Aβ deposition and neurofibrillary tangles induced by hyperphosphorylated tau are two major pathological hallmarks of AD. Therefore, the effects of Beclin-1 overexpression and hUC-MSCs transplantation on Aβ deposition and Tau phosphorylation in mouse brain tissues were detected by immunofluorescence and Western blotting, respectively. As shown in *Fig. 6-A*, obvious Aβ plaques were formed in the brains of AD mice, and transplantation of hUC-MSCs and overexpression of Beclin-1 significantly reduced Aβ deposition. The results of Western blotting revealed that the expression levels of P-tau (Ser396), P-tau (Ser231) and P-tau (Ser235) were all significantly inhibited by transplantation of hUC-MSCs and overexpression of Beclin-1, especially in OE Bec-P15MSCs group *(Fig. 6-B)*. These results suggest that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can effectively reduce Aβ load and Tau hyperphosphorylation in AD mice.

Overexpression of Beclin-1 Combined with Transplantation of hUC-MSCs Promoted Neurogenesis in the Brain of AD Mice

Neuronal loss and neurogenesis in the hippocampus play a critical role in maintaining cognitive function. Therefore, neurogenesis was detected by double immunofluorescence staining of neuron-specific markers. The results showed that double positive staining of EdU$^+$/DCX$^+$ cells and

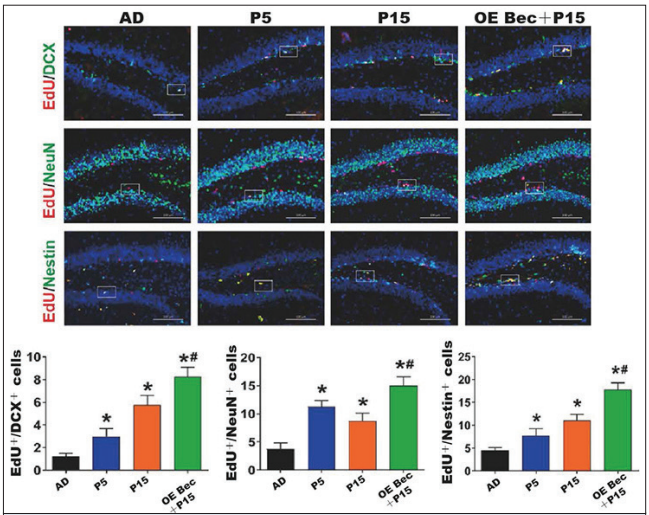

Fig 7. Overexpression of Beclin-1 combined with transplantation of hUC-MSCs promoted neurogenesis in the brain of AD mice. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

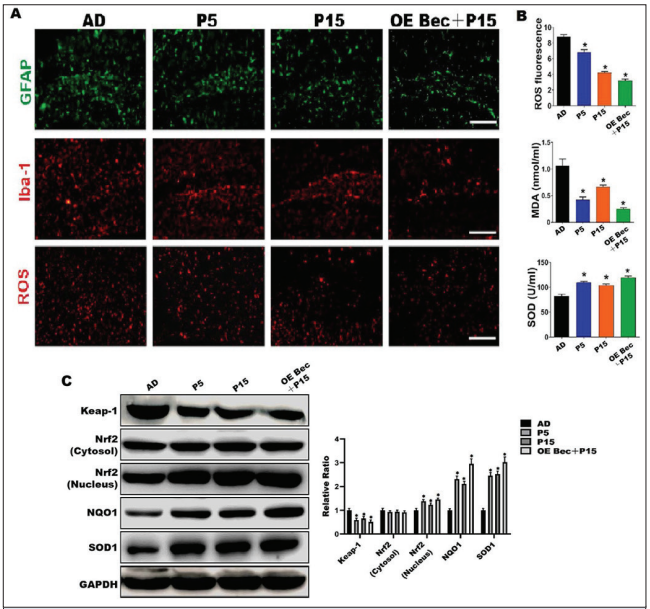

Fig 8. Overexpression of Beclin-1 combined with transplantation of hUC-MSCs activated the Nrf2 signaling pathway. **A:** Immunofluorescence staining of GFAP, Iba-1 and ROS, **B:** Levels of ROS, MDA, and SOD, **C:** Western blotting bands and statistical analysis histograms. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

EdU$^+$/NeuN$^+$ cells was significantly increased in OE Bec-P15MSCs group as compared to that in other groups (P<0.05), and the proportion of EdU$^+$/Nestin$^+$ cells was also the highest in OE Bec-P15MSCs group (P<0.05). Beclin-1 overexpression promoted the survival of hUC-MSCs *in vivo* and activated the generation of endogenous neural stem cells (NSCs) *(Fig. 7)*. These results suggest that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can ameliorate neuronal loss and promote neurogenesis in the brain of AD mice.

Overexpression of Beclin-1 Combined with Transplantation of hUC-MSCs Activated the Nrf2 Signaling Pathway

To determine whether overexpression of Beclin-1 can regulate the neuroinflammatory microenvironment in the brain of AD mice, the activation status of hippocampal astrocytes and microglia was detected by GFAP and Iba-1 immunofluorescence staining. Compared with that in AD group, the number of GFAP$^+$ or Iba-1$^+$ cells significantly declined in other groups, and the activation degrees of astrocytes and microglia were the lowest in OE Bec-P15MSCs group (P<0.05). Meanwhile, OE Bec-P15MSCs group had the lowest ROS production and MDA content and the highest SOD activity (P<0.05), suggesting that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can alleviate the activation and oxidative stress of glial cells in AD mice. Moreover, the protein expression of Nrf2 in NQO1 and SOD1 in brain tissues significantly rose, while the expression of Keap-1 was down-regulated in OE Bec-P15MSCs group (P<0.05) *(Fig. 8)*. To sum up, overexpression of Beclin-1 combined with transplantation of hUC-MSCs can reduce the activation and oxidative stress of glial cells in AD mice through activating the Nrf2 signaling pathway.

Discussion

There are many close links between cellular senescence and oxidative stress. Oxidative stress responses can induce apoptosis and cell cycle arrest, thus accelerating cell death or depriving cells of normal function [21]. HUC-MSCs are the most widely used stem cells in clinical practice, but they are prone to senescent phenotype after culture over several passages *in vitro* [22], thus limiting their further practical application. In this study, compared with P5 hUC-MSCs, P15 hUC-MSCs exhibited signs of cellular senescence such as significant morphological changes, swelling, decreased proliferation and migration, and enhanced oxidative stress. Rejuvenating aged hUC-MSCs to increase the therapeutic time window and efficacy in regenerative medicine is of clinical application value [23]. In this study, the overexpression of Beclin-1 reduced the proportion of SA-β-gal$^+$ cells, inhibited the expression of senescence-related proteins, promoted the proliferation and migration of hUC-MSCs, and rejuvenated aged hUC-MSCs.

Nrf2 is a key transcription factor that regulates cellular oxidative stress and inflammatory response, and maintains stem cell function and nerve regeneration [24]. Under normal circumstances, Nrf2 is continuously ubiquitinated by Keap-1 and degraded *via* the ubiquitin-proteasome pathway. Under oxidative stress, however, Nrf2 translocates to the nucleus and binds to antioxidant response element sites to activate the expression of downstream genes

such as HO-1, SOD-1 and NQO-1 [25]. In this study, the overexpression of Beclin-1 significantly reduced the expression of Keap-1 in aged hUC-MSCs and enhanced the expression of Nrf2, NQO1 and SOD1 in the nucleus, indicating that Beclin-1 activated the activity of Nrf2 signaling pathway. In addition, Beclin-1-overexpressing hUC-MSCs were superior to simple hUC-MSCs in improving the cognitive function, reducing neuronal loss, and promoting neurogenesis in AD mice. Pickford et al.[26] demonstrated the protective role of Beclin-1 in AD. Additionally, Beclin-1 depletion in APP transgenic mice disrupts autophagy, aggravates Aβ pathology, and promotes neurodegeneration [27]. These findings verify the ability of Beclin-1 to rejuvenate aged hUC-MSCs both *in vivo* and *in vitro*, but the exact underlying mechanism still needs further investigation.

Despite growing evidence that stem cell therapy is a promising strategy for treating AD, the chronic pathological microenvironment in the brain is not conducive to the survival of transplanted stem cells [28]. Therefore, it is necessary to develop new approaches to improve the efficacy of stem cell therapy. Wang et al. found that the neuroprotective effect of hUC-MSCs plus resveratrol on AD mice was better than that of single treatment, and MG53 protected hUC-MSCs from LPS-induced inflammatory injury and enhanced their effect on LPS-treated C57/BL6 mice [29]. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs significantly alleviated the cognitive impairment and depression-like behavior in AD mice, indicating that this combination therapy had a better therapeutic effect than the transplantation of hUC-MSCs alone.

Aβ deposition and Tau phosphorylation are two main pathological features of AD. Esmaeilzade et al.[30]reported that Aβ plaques formed in the brain of APP/PS1 mice at 3 months of age. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs significantly reduced Aβ deposition and Tau hyperphosphorylation in AD mice. Likewise, the reduced levels of Aβ plaques and Tau phosphorylation have been reported to benefit both young and aged AD-like animals [31,32]. These results suggest that Beclin-1 and hUC-MSCs can effectively alleviate the pathological changes in AD through a synergistic effect. Aβ-induced neuronal loss is the main cause of cognitive impairment in AD [33]. This study showed that the combination therapy relieved the neuronal loss and preserved the neuronal viability in AD mice.

In addition, exogenous stem cell transplantation or endogenous NSC activation can promote neurogenesis [34]. In this study, the overexpression of Beclin-1 raised the survival rate of hUC-MSCs in the brain of AD mice. The overexpression of Beclin-1 combined with transplantation of hUC-MSCs increased the number of EdU^+/DCX^+, $EdU^+/NeuN^+$, and $EdU^+/Nestin^+$ cells in the hippocampus, suggesting that this combination therapy can also promote neurogenesis by activating endogenous NSCs. Besides, activated glial cells and oxidative stress can damage the structure and function of neuronal cells, and overactivated microglia and astrocytes can induce persistent chronic neuroinflammation, leading to neurodegeneration [35]. Oxidative stress induced by excessive ROS contributes to the senescence of MSCs. Nrf2 is one of the key molecular pathways regulating senescence, inflammation and oxidative stress [36]. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs inhibited glial cell activation and oxidative stress in AD mice by increasing the expressions of Nrf2, NQO1 and SOD1, demonstrating that Beclin-1 promoted the neuroprotective effect of hUC-MSCs on AD mice at least partly by targeting the Nrf2 signaling pathway.

In conclusion, the overexpression of Beclin-1 delays the aging process of hUC-MSCs *in vitro* and enhances the therapeutic effect of hUC-MSCs on AD mice in improving cognition, reducing Aβ deposition and Tau hyperphosphorylation, and increasing neurogenesis.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (P. Ma) on reasonable request.

Financial Support

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Ethical Approval

The human experiment regarding the collection of umbilical cord tissues has been approved by the ethics committee of Xuzhou First People's Hospital on January 4th, 2021 (approval No. XFPH202101003). All animal experiments have been approved by the animal experiments ethics committee of Xuzhou First People's Hospital on March 8th, 2021 (approval No. XFPH-dong202103004).

Competing Interests

There is no conflict of interest.

Authors' Contributions

Li Shao and Pengju Ma designed this study and significantly revised the manuscript; Qing He, Xin Du, Qing Li, Siyuan Yang and Chen Dong performed this study and prepared this manuscript. All authors have approved the submission and publication of this manuscript.

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Research Article

Molecular Analysis and Associated Risk Factors of *Theileria annulata* in Cattle from Various Zones of Balochistan, Pakistan

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Abstract

Theileriosis is a protozoan parasite that is transmitted by ticks and infects a wide range of animals worldwide. This study aims to assess the molecular prevalence and related risk factors of theileriosis in Balochistan, Pakistan. Standard microscopy methods, polymerase chain reaction (PCR), 18S small subunit ribosomal RNA gene sequencing, and phylogenetic analysis were used. For this purpose, a total of 408 blood samples were collected from tick-infested cattle in Zhob, Loralai, and Quetta districts of Balochistan, Pakistan. Microscopy and subsequent PCR analysis confirmed the highest prevalence of *Theileria annulata* in Loralai district (11.76% and 12.75%), followed by Zhob district (11.27% and 12.25%), and Quetta district (8.34% and 9.56%), respectively. Moreover, the prevalence of *T. annulata* was higher in young cattle (85.82%), followed by female cattle (58.87%), and exotic crossbred cattle (33.33%) in the study area. However, various variable such as sex, area, and breeds of the cattle were not significantly correlated ($P>0.05$) with the presence of *T. annulata,* except for the age of animals ($P<0.05$). In addition, sequencing and phylogenetic analyses revealed that the isolated *T. annulata* was closely related to the isolates from Türkiye, Italy, Egypt, Iran, and Pakistan. Hence, these findings will contribute to the development of more effective control strategies for theileriosis in the cattle population of Balochistan, as well as in Pakistan on a broader scale.

Keywords: Balochistan, Cattle, Phylogeny, Prevalence, Risk factors, *Theileria annulata*

Introduction

Agriculture is the backbone of Pakistan's economy, with livestock contributing 14.04% to the country's overall national GDP [1]. In Balochitsan province, 85% of population is directly or indirectly linked to livestock, which contributes over 45% to the province's GDP [2]. The cattle population in Pakistan is around 51.5 million, playing a significant role in animal husbandry for milk, meat, and hides [3]. Livestock is the main source of livelihood for the local people in Balochistan, especially cattle breeding, which serves as a key source of income [4]. A recent study has revealed that Ixodid ticks have a substantial impact on infesting livestock and transmitting a wide range of pathogens in Pakistan. Lack of farmer awareness about the threat of theileriosis and the presence of tick vectors on pasture and animals are key factors leading to severe economic losses in the livestock industry [5].

Ixodidae ticks can transmit parasitic, viral, bacterial, and rickettsial diseases to animals and humans, and affect the host in several ways, including reduced body weight, milk production, reproduction, and reduced quality of hides [6]. Similarly, tropical theileriosis in cattle is caused by a tick-borne haemoprotozoan parasite *Theileria annulata*, which is transmitted by ticks of the genus *Hyalomma*, and the Ixodidae [7]. Tropical theileriosis is frequently reported from North Africa, Europe, Middle East, India, and Central Asia, including Pakistan [8]. In Pakistan, *T. annulata* is transmitted by *Hyalomma anatolicum* to bovine animals [1]. While, *T. annulata* infection is characterized by high fever, anorexia, enlargement of

superficial lymph nodes, anemia, hemoglobinuria, ocular-nasal discharges, cessation of rumination, and diarrhea in cattle [9]. Henceforth, *T. annulata* not only affects the health of animals but also causes serious economic losses in terms of vaccination, treatment, reduction in milk yield and live weight of the animal, while it also increases the inter-calving interval, delay in the age of maturity and causes up to 70% mortality in infected animals [10].

In Balochistan, parasitic infections are the most prevalent among all other animal diseases [11]. This could be attributed to the poor healthcare system, open grazing practices, and inadequate animal management, resulting in significant economic losses [11]. In addition, there is scarce information on the molecular identification of parasitic species in the cattle population of Balochistan. Therefore, this study was designed to investigate and characterize the molecular prevalence of theileriosis and its associated risk factors in different zones of Balochistan, Pakistan. The aim is to develop comprehensive control and therapeutic strategies for theileriosis infection in cattle.

Material and Methods

Ethics Statement

This experimental design was duly approved by departmental ethics committee and directorate of advanced studies, University of Veterinary and Animal Sciences (UVAS), Lahore (ID: DAS/2053).

Study Area, Study Animals and Blood Sampling

A total of 408 blood samples were collected aseptically from tick-infested cattle in Balochistan between July 2021 and November 2022. The sampling was conducted randomly, irrespective to the sex, age, and breed of the cattle, from three different zones in Balochistan, Pakistan. Briefly, 136 blood samples were collected from the 1st zone, Suleiman mountainous region "Zhob", while 153 blood samples were collected from the 2nd zone, Northern highlands "Loralai",

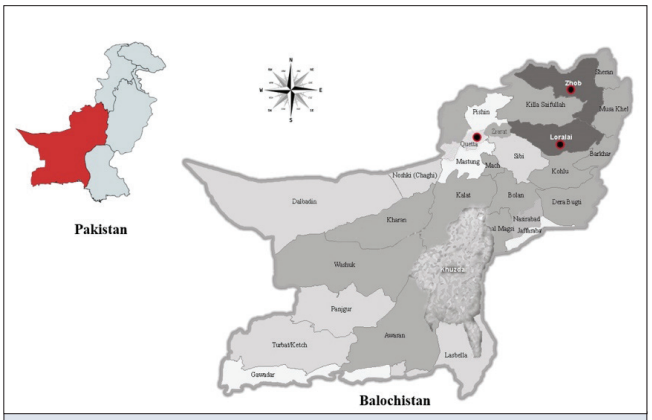

Fig 1. A map created by Mapinfo software, showing the sampling sites; Zhob, Loralai, and Quetta in the Balochistan Province, Pakistan

and 119 blood samples were collected from the 3rd zone, Kachhi basin region "Quetta/Sibi" (Shoaib et al.[12]). The sampling area is depicted in *Fig. 1.* The sample size was determined using the formula provided by Thrusfield;

$N = [Z^2p\ (1 - p)]/\ d^2$

$N= (1.96)^2*0.5(1-0.5)\ /\ (0.05)^2 =384$

Where, N is the desired sample size, P is the predicted prevalence, and d is the 5% precision level.

As mentioned above, the sample size was determined to be 384. However, a total of 408 blood samples were collected in the study area to increase the level of accuracy. A standard performa was used to collect relevant details, such as, age, breed, area, and sex, pertaining to various risk factors [13]. Foe each cattle, five (5) mL of blood was drawn from the jugular vein and placed in vacutainers with EDTA. The blood samples were transported in a cold chain to the Parasitology Laboratory of UVAS, Lahore, and stored at -20°C till further analysis.

Microscopic Analysis

Microscopic analysis was conducted by preparing thin blood smears from the collected samples. The smears were preserved using absolute ethanol and stained with the field's stain A and the field's stain B, as previously described by [14]. Subsequently, the smears were observed under a compound microscope, Olympus CX21 (Olympus, Tokyo, Japan), at a magnification of 100x to detect the presence of *Theileria.*

DNA Extraction and Molecular Analysis

Genomic DNA was extracted from the blood samples using a DNA extraction kit (Thermo Fisher; Cat. No: FABGK001-2), following the manufacturer's instructions. The purity and concentration of extracted DNA were measured using a NanoDrop at 260/280 nm and stored at -20°C for further PCR analysis.

All of the extracted DNA samples were subsequently subjected to PCR, targeting the *T. annulata* cytochrome b gene fragment using specific primers, as previously described by Farooqi et al.[8]. The forward primer used was 5'-ACTTTGGCCGTAATGTTAAAC-3', and the reverse primer was 5'- CTCTGGACCAACTGTTTGG-3', resulting in an amplification size of 450 base pairs.

For each PCR sample, a 20 µL reaction mixture was prepared, consisting of 2 µL of extracted DNA, 25 pmol of each primer, 200 mM of each dNTP, 5 µL of 10X PCR buffer, and 1.5 mM MgCl2 (Promega, Madison, WI, USA), and 1.5 U of Taq DNA polymerase. The first phase of the PCR reaction cycle invovled an initial denaturation step at 94°C for 5 min, followed by thermal degradation for 40 cycles at 94°C for 35 sec, annealing at 51°C for 35

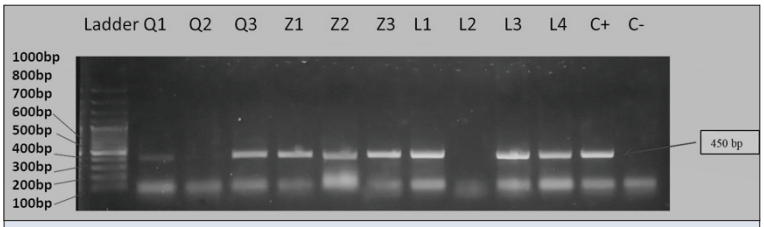

Fig 2. PCR positive products were examined using a gel electrophoresis system to identify *Theileria* species in cattle specimens from three different zones of Balochistan, Pakistan (n=408). A molecular weight marker of 1000 base pairs is visible on the lane ladder. Lane C+ve displays the positive control *(Theileria. annulata* clinical isolate), while Lane C-ve displays the negative control. The other lanes represent the results of the positive and negative samples

sec, and extension at 72°C for 35 sec. The final elongation was performed for 10 min at 72°C, followed by a hold step at 4°C. Each PCR run included a positive control (lab-isolated clinical isolate) and a negative control (sterile water). Positive bands were confirmed using gel electrophoresis and then trimmed for sequencing analysis.

Sequencing Analysis

The PCR bands testing positive for haemoparasites (constituting 50% of the bands positive for *T. annulata*-specific primers) were excised from a 1.5% agarose gel and purified using a gel extraction kit (WizPrepTM; Ref. W70150-300), following the manufacturer's instructions. The purified DNA samples were sent to CELEMICS Product & Services in South Korea for sequencing. A phylogenetic tree was constructed using Mega 11 software with a maximum likelihood algorithm and bootstrapping at 1000 replications.

The PCR bands testing positive for haemoparasites (constituting 50% of the bands positive for *T. annulata*-specific primers) were excised from a 1.5% agarose gel and purified using a gel extraction kit (WizPrepTM; Ref. W70150-300), following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted using SPSS version 20.00. The prevalence of theileriosis was compared to independent variables such as sex, age, area, and breed using the chi-square (χ^2) test. A P-value <0.05 was considered statistically significant, and a 95% confidence interval was established.

Results

Molecular Epidemiology of Theileriosis in cattle

The results of this study showed an overall 34.56% (141/408) PCR-based prevalence of theileriosis in cattle from 3 different zones of Balochitsan, Pakistan i.e. Loralai having a prevalence of 12.75%, Zhob with 12.25% and Quetta with 9.56% *(Fig. 2)*. However, microscopic examination indicated a prevalence of 31.37% (128/408),

Table 1. *Results of the microscopic identification of Theileria species in cattle from three different zones of Balochistan, Pakistan (n=408)*

Sample No.	Districts	Positive n (%)	Negative n (%)	Total n (%)
1	Quetta	34 (8.34)	85 (20.83)	119 (29.17)
2	Zhob	46 (11.27)	90 (22.06)	136 (33.33)
3	Loralai	48 (11.76)	105 (25.74)	153 (37.50)
Total		128 (31.37)	280 (68.63)	408 (100)

Table 2. *PCR based identification of the Theileria species in cattle from 3 different zones of Balochistan, Pakistan (n=408)*

Sample No.	Districts	Positive n (%)	Negative n (%)	Total n (%)
1	Quetta	39 (9.56)	80 (19.61)	119 (29.17)
2	Zhob	50 (12.25)	86 (21.08)	136 (33.33)
3	Loralai	52 (12.75)	101 (24.75)	153 (37.50)
Total		141 (34.56)	267 (65.44)	408 (100)

suggesting that the PCR method is more sensitive compared to microscopic identification *(Table 1, Table 2)*. In addition, the presence of intraerythrocytic inclusion

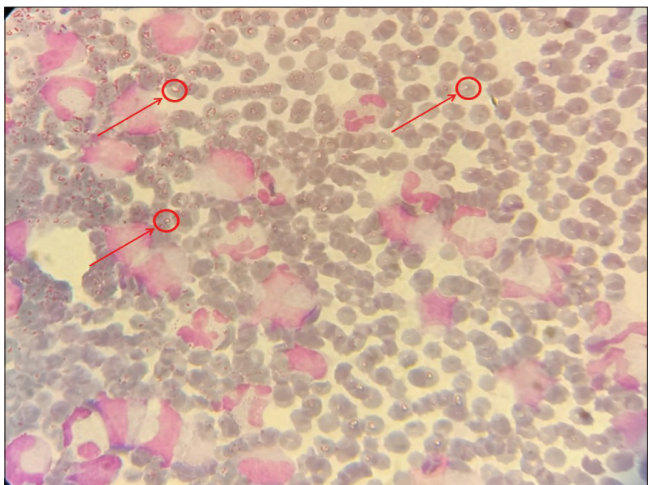

Fig 3. Thin blood smear of cattle samples collected from 3 different livestock zones of Balochistan, Pakistan (n=408), showing typical *Theileria* piroplasms, intraerythrocytic bodies, under oil immersion lens after field staining

Table 3. *Results of chi-square test on various risk factors associated with theileriosis in cattle from three different zones of Balochistan, Pakistan (n=408)*

Parameters		Quetta, (n=39) % Positive	Zhob, (n=50) % Positive	Loralai, (n=52) % Positive	Total (n=408)		Chi-square Value	P-value
					%Positive (n=141)	%Negative (n=267)		
Breed	Sahiwal	20.51	16.00	13.46	16.31	18.73	6.085	0.808
	Friesian	33.34	30.00	23.08	28.38	22.85		
	Red Sindhi	7.69	6.00	9.62	7.80	8.99		
	Cholistani	7.69	4.00	7.69	6.38	11.98		
	Cross Bred	20.51	36.00	40.38	33.33	26.22		
	Jersey	10.26	8.00	5.77	7.80	11.23		
Age	Young (1-2 year)	97.44	82	80.77	85.82	28.84	6.012	0.049*
	Adult (>2 year)	2.56	18	19.23	14.18	71.16		
Sex	Male	56.41	36	34.62	41.13	37.08	5.215	0.074
	Female	43.59	64	65.38	58.87	62.92		
Area	+Ve	9.56	12.25	12.75	34.56	-	0.483	0.786
	-Ve	19.61	21.08	24.75	-	65.44		

** P-value <0.05 was considered significant*

bodies resembling *Theileria* was also confirmed under a light microscope using thin blood smears *(Fig. 3)*.

As shown in *Table 3*, the prevalence of *T. annulata* was higher in young cattle (85.82%), followed by female cattle (58.87%), and exotic crossbred cattle (33.33%) in the studied area. However, various variable such as sex, area, and breeds of the cattle were not significantly correlated (P>0.05) with the presence of *T. annulata,* except for the age of the animals (P<0.05). Exotic cattle breeds, such as Friesian and crossbred, had a higher prevalence of theileriosis compared to local breeds like Sahiwal, Red Sindhi, or Cholistani. This may be attributed to the local breeds being more acclimatized to the conditions in the area. In addition, young animals (0-2 years) were found to be more susceptible to theileriosis compared to adult animals (P<0.05). Female cattle also exhibited a higher prevalence of theileriosis compared to males (P>0.05). These findings suggest that young animals are at a higher risk of theileriosis, compared to adult animals.

Sequencing Analysis of *Theileria* 18S rRNA Gene in Cattle

PCR positive samples were sequenced to analyze the 18S rRNA gene fragments of *Theileria*. The sequences were subjected to analysis using tools such as BLAST & CLUSTAL W alignments. Nucleotide sequences of the *Theileria* 18S rRNA gene were obtained from the NCBI GenBank for comparison. Phylogenetic analysis and BLAST queries revealed a 99% sequence similarity

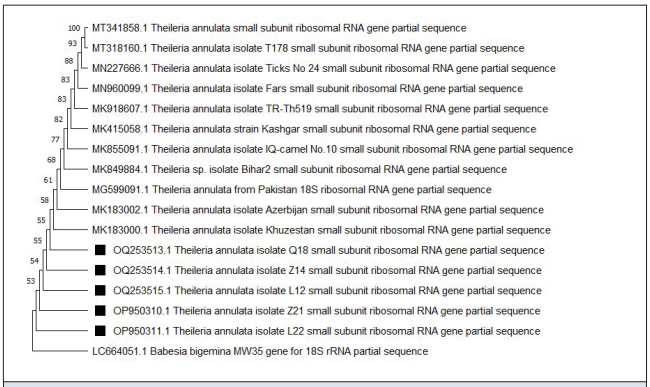

Fig 4. Phylogenetic tree illustrating sequence similarity among samples OQ253513.1, OQ253514.1, OQ253515.1, OP950310.1, OP950311.1, and previously reported sequences of the 18S small subunit rRNA gene of *Theileria annulata*. The partial 18S rRNA gene sequence of *Babesia bigemina* was included as an outgroup

to *T. annulata* 18S rRNA gene from various countries including Pakistan (MT318159), Iran (MN960099), Italy (MT341858), Türkiye (MK918607), and Egypt (MN227666). Notably, some of the sequences obtained in this study have been submitted to the NCBI GenBank *(Fig. 4)*.

Discussion

Tropical theileriosis is a well-known hindrance to the growth of the dairy industry worldwide and causes significant economic losses to the livestock industry [15,16]. However, there is a lack of sufficient and up-to-date molecular epidemiological studies on tick-borne

infections, particularly theileriosis, which hinders the development of preventive and control strategies [16]. In Balochistan, where a majority of the people rely on livestock for their livelihood, tick-borne diseases pose serious risks to the livestock industry [17]. Therefore, this study was designed to investigate the molecular prevalence and relevant risk factors of theileriosis in cattle from three different zones of Balochistan: Zhob, Loralai, and Quetta. Initial examination of thin blood smears from the sampled animals showed intra-erythrocytic bodies of theileriosis under a light microscope, consistent with previous findings Ekoka Mbassi et al.[14], Adehan et al.[18] and Chauhan et al.[19], which also observed similar intra-erythrocytic bodies in the blood smears of the *Theileria* positive animals.

In this study, a PCR-based prevalence of theileriosis was observed in cattle from 3 different zones of Balochitsan, Pakistan i.e. Loralai (12.75%), Zhob (12.25%) and Quetta (9.56%), accounting for a total of 34.56%. These results were in line with a study conducted by Durrani et al.[20], which reported a PCR based prevalence of *Theileria* in cattle from China to be 32.4%. Similarly, the prevalence of *T. annulata* in cattle from North-Western Pakistan was found to be 29.9% [21]. However, microscopic examination revealed a lower prevalence of theileriosis, specifically 31.37% (128/408), compared to PCR based prevalence. This findings aligns with previous reports by Chen et al.[22], Zeb et al.[23], and Zaman et al.[24], suggesting that microscopic identification is less precise and sensitive compared to PCR analysis. Therefore, PCR is considered the preferred tool for identifying blood-born parasites [25-,27].

In the present study, age of the animals was statistically significant risk factor for the occurrence of theileriosis in cattle of Balochitsan ($P<0.05$). However, other risk factors such as sex, area, and breeds of animals were statistically non-significant ($P>0.05$). These results were consistent with the reports of Mohammed-Ahmed et al.[28], Farhan et al.[29], and Kawan [30], suggesting that the occurrence of theileriosis in different zones may be linked with various known or unknown variables. Furthermore, the prevalence of theileriosis was observed to be higher in crossbred and Friesian cattle compared to Sahiwal, Red Sindhi, and, Cholistani, breeds in Balochitsan. These findings were similar to the results reported by Ghafar et al.[31] and Rana et al.[32], which indicated a higher prevalence of *T. annulata* in crossbred and exotic breeds of cattle compared to local breeds. Collectively, these findings indicate that exotic breeds and their crossbreeds face an increased susceptibility to tick-borne diseases [33,34]. This vulnerability may be attributed to inherent resistance, grooming behavior, or other biological factors influencing the interaction between native breeds and ticks. Furthermore, it is crucial to explore environmental conditions, climatic factors, and other ecological parameters that could contribute to a more stable or unstable enzootic state in various areas of Balochistan [33,34].

In addition, this study showed that female and young cattle (0-2 years) appeared to have a higher prevalence of *Theileria* than male and adult cattle in Balochistan. These findings were consistent with the results reported by Aslam et al.[35] and Farooq et al.[36], who also observed a higher prevalence of *T. annulata* in younger animals. However, Valente et al.[37] found no significant correlation between sex and the presence of theileriosis in cattle. On the other hand, Marwaha et al.[38] observed that the prevalence of theileriosis was higher in adults compared to young animals, possibly due to the higher immunity level in young animals caused by fetal hemoglobin in their circulatory system [39]. Moreover, the increased occurrence of theileriosis in younger animals, as observed in this study and others, might be linked to the larger body surface area of adult cattle, their outdoor management, prolonged movement over long distances in search of feed and water. In addition, the hormonal issues in female animals could potentially contribute to the higher prevalence of haemoparasitic infections in female cattle [40].

Finally, BLAST and phylogenetic analysis of the study isolates revealed a 99% sequence similarity with the *Theileria* isolates from Italy, Pakistan, Egypt, Iran, and Türkiye. The 18S ribosomal RNA gene sequence of studied isolates was aligned with similar reference sequences, showing significant variation in the genotype of *T. annulata* from 3 different zones of Balochistan, Pakistan. Similar studies on the sequencing and phylogenetic characterization of *T. annulata* have been conducted by Sisson et al.[41], Gargano et al.[42], and Solomon and Tanga [43], which are closely related to this study. Hence, it is important to highlight that the sequence similarities among globally discovered haemoparasites are primarily due to the export and import of animals [44].

The present study concludes that *T. annulata* is the main species causing theileriosis in the cattle populations of Balochistan, Pakistan. Several risk factors such as sex, area, and breeds of the animals, were not significantly associated with the disease dynamics, except for the age of the animals. Consequently, young animals are more susceptible to suffering from theileriosis. Overall, these results will be beneficial in designing more effective control strategies for theileriosis and provide a baseline for further research to eradicate or decrease haemoparasites in the future.

Availability of Data and Materials

The datasets generated during and analyzed during the current study are available from the corresponding author (K. Ashraf & M.U. Rehman) on reasonable request.

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Conflicts of interest

All other authors declare no conflicts of interest

Ethical Statement

This experimental design was duly approved by departmental ethics committee and directorate of advanced studies, University of Veterinary and Animal Sciences (UVAS), Lahore (ID: DAS/2053).

Author Contributions

KA and MUR contributed to the conceptualization, design, funding and supervision of the study. FK conducted all experiments and wrote the first draft of the manuscript. HA and MA collected, analyzed and interpreted the data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Synergistic and Dose-Dependent Effects of Pinostrobin, Pinocembrin and Pinobanksin on Different Breast Cancer Cell Lines

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Abstract

In this study, the cytotoxic and apoptotic effects of three phenolic compounds highly found in the poplar type propolis; pinostrobin (PS), pinocembrin (PC) and pinobanksin (PB), were investigated individually and in combination on hormon-positive (MCF-7) and triple negative (MDA-MB-231) breast cancer cell lines and fibrocystic breast epithelium (MCF-10A) as control. Assessment of cytotoxicity and apoptosis were performed with WST-1 and Annexin V-7AAD assays, respectively. All statistical analyses including the two-way ANOVA and multiple-t-test were performed using GraphPad Prism software. Individually, PB ($P<0.0001$), PS ($P<0.0001$), and PC ($P<0.05$) demonstrated potent cytotoxic effects at moderate to high doses and late time intervals on MCF-7. PB and PS have been found to have a significant proliferative effect at low doses ($P<0.0001$), however, this effect disappeared in higher doses in this cell line. Dual combinations of PB+PC and PB+PS were toxic on MCF-10A, however, dual combination of PS+PC and the triple combination (PB+PS+PC) showed no cytotoxicity until high doses at late time intervals ($P>0.05$). On MCF-7, the triple combination induced cytotoxic/apoptotic effects even with the 25% dose and 50% dose on MDA-MB-231($p<0.0001$). Our findings clearly showed that different combinations of these phenolic substances can have synergistic cytotoxic effects and even hormetic effects in non-tumorogenic cells.

Keywords: Breast cancer cell line, Cytotoxicity, Pinobanksin, Pinocembrin, Pinostrobin, Propolis

Introduction

Breast cancer (BC), a heterogeneous disease, comprises 4 main molecular subtypes with different gene expression profiles and clinical outcomes [1,2]. Luminal A and B subtypes are characterized with estrogen receptor (ER) positivity, HER2+ with the over amplification (over-expression) of HER2 and triple-negative with the lack of ER, progesterone receptor (PR) expressions and HER2 overexpression [3]. The hormone receptor positive luminal types are the most commonly diagnosed types with making up 70-75% of all BCs [4]. The main medical treatment approach for ER+ BCs is endocrine treatment [5], yet recurrences occur in 20-30% of the cases [6]. Moreover, the most aggressive type, triple negative BCs constitute 10-20% of all invasive BCs. There is no targeted therapy available against triple negative BCs, thus, only the conventional chemotherapy is used, which has a poor response rate in these cases [7]. Therefore, supporting approaches are investigated in the treatment of BC.

The lack of targeted therapy and high recurrence rates made many researches focus on different complementary approaches. Propolis, one of the most focused substances among these, is a natural product produced by honeybees from substances collected from various plants. It is

widely used since the ancient times, generally owing to its anti-bacterial, anti-viral properties, and more recently further for its anti-oxidant, anti-inflammatory and anti-tumoral properties [8-10]. Among the active components of propolis, bioflavonoids emerge as the most prominent. Flavonoids, secondary metabolites of plants, are highly found in the poplar type propolis, including pinocembrin and pinobanksin [11-14]. Pinostrobin (PS), which was discovered in pine *(Pinus strobus)* wood content 60 years ago, is a flavanon, a subtype of flavonoids. It can be found in galangal, honey and propolis. It has been demonstrated that pinostrobin shows significant biological activities such as anti-proliferative [15,16], anti-inflammatory [17], anti-oxidant [18], and anti-aromatase [19] effects on various cancer cell lines, inhibits HIV-1 protease [20], and shows anti-ulcerative activity [21]. Moreover, by decreasing the Alzheimer's disease related amyloid peptide activity and calcium overload inhibits the mitochondrial pathway related to apoptosis [22]. The cytotoxic effect of pinostrobin may be related to topoisomerase inhibition [23] and its anti-aromatase effect in hormone-dependent cancers [19]. However, like many other flavonoids, pinostrobin also shows low water-solubility which creates a significant limitation for its use in pharmaceutical applications.

Pinobanksin (PB), a member of the dihydroflavonol subtype of flavonoids, is found in Pinus resinosa tree trunk [24], sunflower honey and propolis [25]. It has been shown that besides reducing Fe (II)- induced lipid peroxidation, it also shows antioxidant activity by inhibiting mitochondrial membrane permeability transmission [26]. Some PB derivatives are shown to be apoptosis inducing compounds [27], however, their apoptotic effects vary among cancer cell lines. While pinobanksin-3-acetate (PB3A) application to HCT-116 colon cancer cell lines have shown to induce apoptotic and anti-carcinogenic effects [28], it showed no effect on A549 lung adenocarcinoma cells [24].

Application of pinocembrin (PC), a member of the flavanon subtype, induced Bax-dependent apoptosis in HCT-116 colon cancer cell line [29]. PC showed protective effects against LPS related inflammation both *in vitro* and *in vivo* [30]. Furthermore, in a study conducted on diabetic mice, it was demonstrated that it reduces reactive oxygen radical and inflammatory agent levels [31]. Moreover, it was found to be a BC resistance protein 2 (ABCG2) inhibitor [32]. Lastly, it was shown to inhibit TGF-β1 induced cell invasion and migration in retinoblastoma cells in non-cytotoxic concentrations [33]. In the same study, it was reported that PC decreased vimentin, N-cadherin and integrin expressions, and inhibited focal adhesion kinase (FAK) and p38α signal activation via inducing the down-regulation of pTGF-β1 induced metalloproteinase enzyme mRNA and protein levels.

Cancer may relapse or cells may generate multiple drug resistance, therefore, current treatment approaches are frequently inadequate to achieve a complete cure. To overcome, novel, less aggressive approaches are needed. In many studies, various plant-based active substances attracted attention and were found promising. Although propolis is one of the most focused substances for this purpose, the propolis content varies according to seasonal and regional flower diversities, thus the cytotoxic and biological properties of propolis differ depending on its content.

Therefore, in this study, it was aimed to determine the cytotoxic and apoptotic effects of PS, PB and PC, substances found in poplar type propolis in varying levels, individually and in combination on a human breast cancer cell line MCF-7 and a human fibrocystic breast epithelial cell line, MCF-10A as a control. Moreover, in order to evaluate the effects of the triple-mixture on the most aggressive BC type, the MDA-MB-231, triple negative BC cell line was utilized.

Material and Methods

Cell Culture

In this study, MCF-7 cell line representing hormone positive (ER/PR(+)), triple negative (ER/PR(−), HER2(−)) MDA-MB-231 breast cancer cell lines and MCF-10A cell line representing fibroadenoma breast epithelial as control were used. All cell lines purchased from the American Type Culture Collection (ATCC) (Rockville, Maryland, USA) were maintained in their recommended mediums and all supplemented with 1% glutamine and 1% penicillin and 10% fetal bovine serum (FBS) (Biochrome, Berlin, Germany).

Preparation of Test Solutions of Phenolics

The main stock solutions were formed by dissolving the HPLC >95% purity PC (No: PHL80061), PB (No: 68530) and PS (No: 38790) in 60% ethanol purchased from -Aldrich (Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany). Then the doses to be applied in the experiment were diluted with the medium containing 3% FBS. Doses of pinobanksin (1.25, 2.5, 5, 7.5, 12.5, 15, 20, 25 µg/mL), pinostrobin (1.25, 2.5, 5, 7.5, 12.5, 15, 20, 25, 37.5, 50 µg/mL) and pinocembrin (2.5, 7.5, 15, 22.5, 30, 37.5 µg/mL) were applied to the cells to determine the effects at different time intervals (24th, 48th, and 72nd h). The substances dissolved with the sonicator were filtered through a 0.22 µm diameter filter before application.

First of all, the individual half-maximal inhibitory concentration (IC50) values of the substances were determined, then, in order to prepare the dual combinations and triple mixtures, they were brought together as in the final total mixture, the concentrations of every single

substance were similar to their IC50 values (MCF-7), which is stated as 100%. After that, the 25%, 50% and 75% doses were prepared with following dilutions. All four doses of the dual combinations and triple mixture were applied to MCF-7 and MCF-10A, in addition, only the triple mixture was applied to MDA-MB-231.

Cell Viability Assay

In vitro cytotoxicity studies were performed using the WST-1 test, which is more sensitive and easier than other tetrazolium salt based cell viability tests. Cells were trypsinized and plated in 96 well plates $1x10^4$ cells per well. Determined doses of PS, PB and PC were administered to cells both individually and in combination after 24 h for cell attachment and differentiation. WST-1 reagent was applied and the plates were incubated for 2 h in the dark prior to 24, 48 and 72 h following substance administration. Plate absorbances were measured with a multi-plate reader (Thermofisher Sci., Waltham, MA, USA) at 450 and 620 nm wavelengths at the 24th, 48th, and 72nd h.

Apoptosis Assay

Annexin V and Dead Cell Assay was performed utilizing Muse™ Cell Analyzer (Millipore Corporation, Merck, KGaA, Darmstadt, Germany). Per well, 250,000 cells were seeded into 6 well plates and 24 h were allowed for cells to attach and differentiate. Cells were treated with the triple combination mixture containing PB, PS and PC with the specified dose, and at the following 48th h they were treated with Annexin V and Dead Cell Reagent (7-AAD) (Merck, KGaA, Darmstadt, Germany) incubated for 20 min. After incubation, each sample was analyzed utilizing the Muse™ Cell Analyzer device. Dead, late apoptotic, early apoptotic and viable cells were determined using 3 biological replicates.

Statistical Analysis

Power analysis was performed using PS Power and Sample Size Calculation (PS) program *(http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize)* [34]. The sample size was estimated using the data published in the article by Wiyono et al.[35], regarding the two groups, MCF-7 and MDA-MB-231, treated with the same doses of pinostrobin. Assuming the difference of 9.948 between the two groups and an standart deviation of 3.366, the sample size estimated for a t-test (P<0.05 and power=0.8) was in a total of 6 samples (3 samples per group).

WST-1 finding were evaluated utilizing Dunnet's multiple comparisons test and calculations of the IC50 values were performed on the GraphPad Prism version 9.5.0 software (San Diego, CA, USA). Moreover, Annexin-PI evaluations were conducted using two-way ANOVA Sidak's multiple comparisons test. A P-value less than 0.05 was considered significant. The values represent the mean±standart deviation of three independent experiments, each carried out in duplicate.

Results

The Individual Proliferative Effects of PB, PS and PC on the MCF 7 and MCF 10A Cell Lines

Cytotoxic effects of PB, PS and PC on MCF-10A and MCF-7 are given in *Fig 1*. PB was found ineffective on MCF-7 at the 24th and 48th h (P>0.05), but on the 72nd h starting from the dose of 15 µg/mL cytotoxicity was observed (P<0.0001). On MCF-10A, a statistically significant proliferation was observed at the 24th and 72nd h with the doses of 1.25 and 2.5 µg/mL (P<0.0001). Starting from 5 µg/mL anti-proliferative effect was observed at the 48th and 72nd h (P<0.05) *(Fig. 1)*.

In the MCF-10A cells, the IC50 values of PB at 24 and 48 h were undetectable as it did not show more than 50% inhibition in this cell line at all doses, while the 72nd h value was determined as 17 µg/mL.

PS was found ineffective with low doses (1.25 and 2.5 µg/mL) on MCF-7 (P>0.05), while it showed cytotoxic effects at all the time intervals starting from the dose of 12.5 µg/mL (P<0.0001). At lower doses (1.25, 2.5, 5 and 7.5 µg/mL) a proliferative effect was observed on MCF-10 at all 3 h (P<0.0001), however, this proliferative effect disappeared after the 12.5 µg/mL dose *(Fig. 1)*. The IC50 values were determined as 20.15 µg/mL, 20.22 µg/mL and 19.53 µg/mL at the 24th, 48th, and 72nd h respectively.

Although no significant cytotoxicity was observed in MCF-10A in all hours with the PC treatment (P<0.05), an increased anti-proliferative effect with the dose was found in MCF-7 starting from the dose of 15 µg/mL at the 48th and 72nd h (P<0.05) *(Fig. 1)*. The IC50 values were determined as 20.74 µg/mL, 20.67 µg/mL and 19.24 µg/mL at the 24th, 48th, and 72nd h respectively.

Effects of Combined Applications of PS, PB and PC on Cell Proliferation in MCF-7 and MCF-10A Cells

WST-1 assay demonstrated that PB and PC in combination have an anti-proliferative effect on MCF-10A at all doses at all of the time intervals (P<0.0001), but only at the 48th and 72nd h a significant cytotoxic effect was observed on MCF-7 (P<0.0001). Cytotoxic effects were similar in MCF-10A cells treated with PB and PS at all doses and all three h (P<0.0001). In MCF-7 cells, no significant cytotoxicity was observed at the 24th h (P>0.05), but approximately 80% of the cells (P<0.0001) were dead at the 48th h at every 4 doses and 50% at the 72nd h (P<0.0001). As a result of the combined application of PS and PC, a significant proliferation was observed in MCF-10A at a dose of 25% at the 24th h (P=0.0066) and a dose-dependent cytotoxic

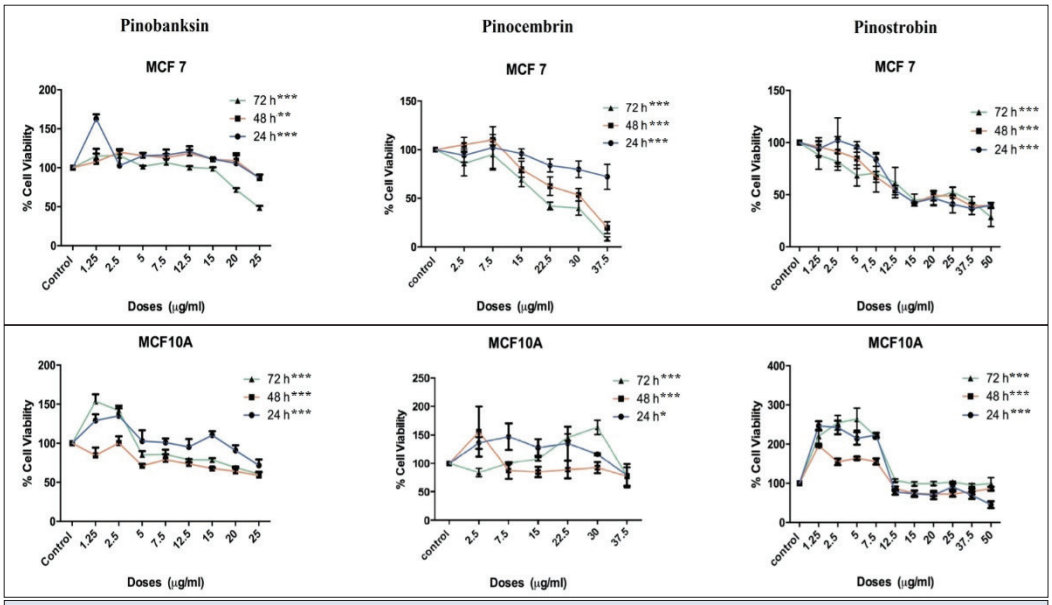

Fig 1. Cellular cytotoxicity of all three substances individually on MCF-10A and MCF-7. Detailed statistical findings are presented in results section. *P<0.05, **P< 0.01, ***P< 0.0001 compared to the untreated group

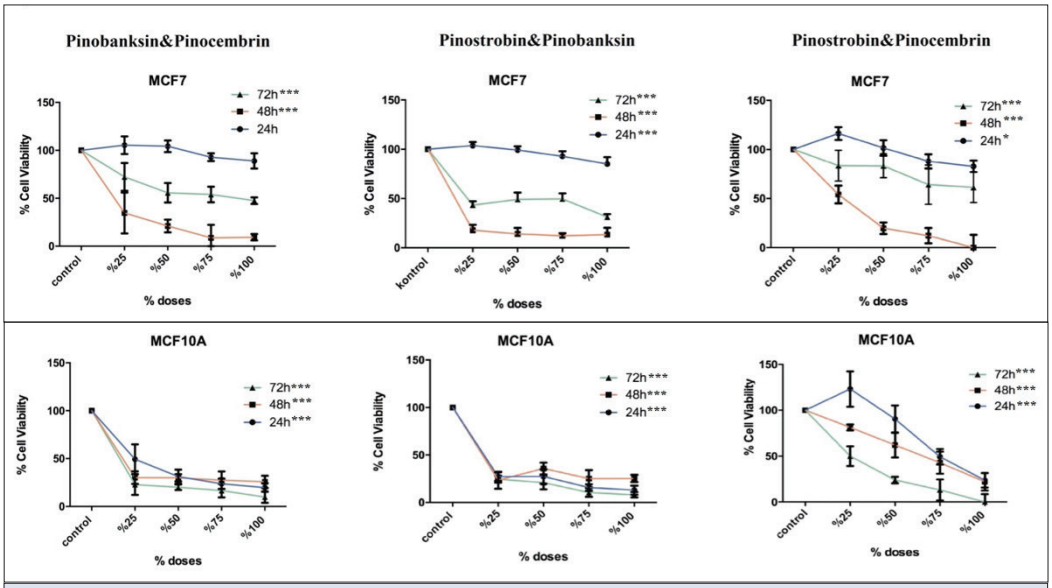

Fig 2. Cellular cytotoxicity of all dual combinations on MCF-10A and MCF-7. Detailed statistical findings are presented in results section. *P< 0.05, **P<0.01, ***P< 0.0001 compared to the untreated group

effect was observed starting from this dose. In MCF-7, dose-dependently increased cytotoxicity was observed at all doses at the 48th h, while a significant cytotoxic effect was only observed at 75% and 100% doses at the 72nd h (P<0.0001) *(Fig. 2)*.

PB, PS and PC triple mixture induced a significant dose dependent cytotoxic effect on MCF-7 cells at all doses starting from 25% at all 3 h and became severe with the 50% dose (P<0.0001). On MCF-10A cells, a dose dependent cytotoxicity was observed, with a milder effect at the 24th h (P<0.0001), and more dramatic effect at higher doses at the 75% and 100% doses at late time intervals (48th and 72nd h) (P<0.0001). Moreover, on MDA-MB-231, a significant cytotoxicity was detected starting from the 50% dose at all 3 h (P<0.0001). A preferred toxicity was observed with the 50% dose of the triple mixture being more effective in MCF-7 cell line and MDA-MB-231 than MCF-10A. Therefore, these conditions were selected for the determination of apoptotic and necrotic death *(Fig. 3)*.

Flow Cytometric Analysis

Fourty-eight h following the treatment of the 50% dose of the triple mixture on MCF-7 and MCF-10A cells, each cell line was compared with its own untreated controls. Annexin V-7AAD analysis findings are given in *Table 1*

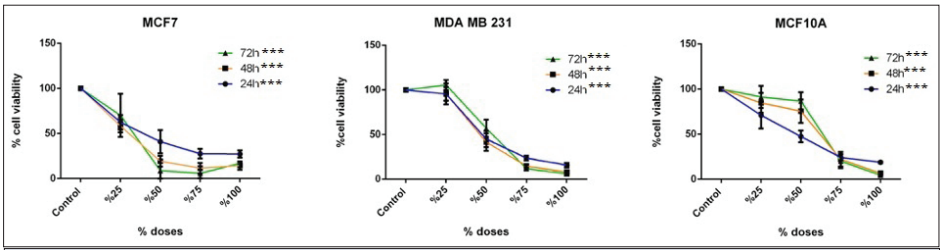

Fig 3. Cellular cytotoxicity of the triple mix on MCF-10A, MCF-7 and MDA-MB-231. Detailed statistical findings are presented in results section. *P<0.05, **P<0.01, ***P<0.0001 compared to the untreated group

Table 1. *Statistical Analysis of the Annexin-V/7-AAD of the triple mixture on MCF-10A, MCF-7 and MDA-MB-231*

Cell Types	MCF-7		MDA- MB- 231		MCF-10A	
	Mean+SD	P Value	Mean+SD	P Value	Mean±SD	P Value
Live (%)	58.24±2.86	< 0.0001	63.41±2.63	< 0.0001	84.13±2.65	0.0112
Early Apoptotic (%)	19.52±1.75	< 0.0001	24.92±1.56	< 0.0001	10.54±1.36	0.0011
Late Apoptotic (%)	21.26±2.22	< 0.0001	10.04±1.09	0.0001	4.14±1.46	0.8349
Debris (%)	0.99±0.07	0.9985	1.63±0.79	> 0.9999	1.19±0.39	> 0.9999
Total Apoptotic (%)	40.77±2.85	< 0.0001	34.96±2.04	< 0.0001	14.68±2.5	0.0125

Percentage of apoptotic cells post treatment with 50 % doses of triple mixture for 48 h. Values were mean ± SD from three independent experiments (n = 3)

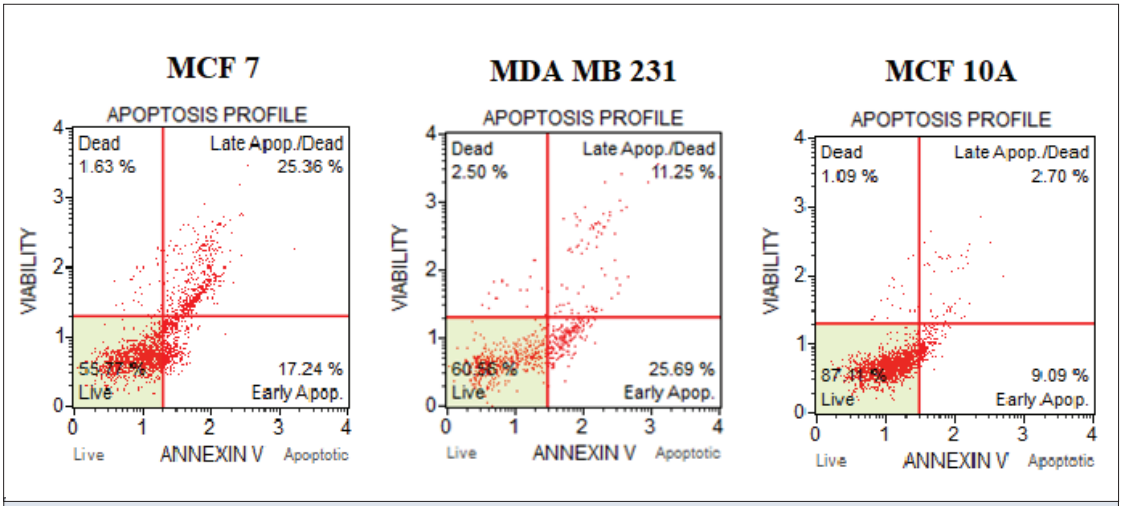

Fig 4. Annexin-V/7-AAD analysis of the triple mixture on MCF-10A, MCF-7 and MDA-MB-231

and *Fig. 4*. The triple mixture induced significant apoptotic death on MCF-7 and MDA-MB-231 cells but not on MCF-10A cells.

Discussion

Propolis is produced by honeybees basically to protect and sterilize their hives from different plant exudates, therefore contains a variety and high levels of phenolic compounds, which attracted many researchers especially this past decade [1,9]. The content, types and efficiency on different diseases have been the main focus. Many studies demonstrated its numerous significant biological effects such as anti-inflammatory, anti-bacterial, anti-viral, anti-fungal, anti-oxidant anti-cancer effects and cardioprotective and hepatoprotective activity of this amazing bee product [8-10]. Studies have shown the anticancer effects of propolis on BC, however, as has been shown before that not every type of propolis may show these effects. The anticancer activity of propolis on BC can generally vary depending on the propolis content and breast cancer type [10,36]. In the presented study, based on the fact that this difference is related to the content of propolis, the effects of PB, PC and PS, compounds highly found in especially poplar type propolis [37], was studied individually and in combination on the most common BC type, hormone responsive (MCF-7) and fibrocystic breast epithelium (MCF-10) as control, *in vitro*. In addition, the triple combination was also investigated on the most aggressive BC type, triple negative (MDA-MB-231)

in order to see its cytotoxic effects, since its treatment approach and efficiency is very limited.

PB was only found effective on MCF-7 at the late time period (72nd h). On the other hand, it showed a proliferative effect with low doses on MCF-10A. In the literature, this incidence is called '*hormesis*' and defines the biphasic behavior with in most cases stimulating effects on proliferation at low doses and proliferation inhibitory effects at high doses. Stress inducers such as heat, dietary restrictions, radiation and various phytochemicals are known to possibly generate the hormetic effect [38,39]. The molecular mechanism of the hormetic effect is thought to be as cells that undergo such stress use all the proteins available to proliferate before being unable to produce more vital proteins. At this point, it can be interpreted as a response of cancer cell against the apoptotic effect of the substance, first by stimulating growth but then not being able to overcome the cytotoxicity/stress. This study is the first to demonstrate the hormetic behavior of PB on MCF-10A cells.

In the study conducted by Xuan et al.[40], PB isolated from Chinese propolis was ineffective at concentrations up to 160 µM (43 µg/mL) on MCF-7 cells at the 24th and 48th h, while in the same study, PC has been shown to be more effective on MCF-7 cells at a dose of 160 µM compared to PB at the 48th h. Our findings also confirmed the cytotoxic effect of PC on MCF-7 starting from the dose of 15 µg /mL at the 48th and 72nd h.

A significant proliferation was observed in MCF10A cells with a low dose administration of PS, also known as pinocembrin-7-methyl ether. A recent study, on the other hand, demonstrated that the maximum dose of 20 µM (5.4 µg/mL) of PS had no effect on proliferation on MDA MB 231 and MCF 10A cells [41]. However, it was observed that PS decreased migration in MDA-MB-231. According to our findings starting from 12.5 µg/mL PS is cytotoxic on MCF-7 cell lines. Moreover, in a previous study, the IC50 value of a pinostrobin derivative, pinostrobin-chalcone, isolated from *Alpinia mutica* rhizomes in MCF-7 cell line was determined as 7.3 µg/mL [42]. In addition, our study is the first in which PB and PC were applied in fibrocystic breast epithelium MCF 10A. The significant cytotoxic effect was observed at a dose of 25% on MCF-10A cells treated with a combination of PB and PC may indicate that administration in combination inhibited the proliferative effect observed with the PB administration alone, suggesting that these two compounds behave differently in a way that PC may be eliminating the growth stimulating activity of PB. With a dose of 25% more than half of MCF-7 cells were dead at the 48th h. Interestingly, PS and PB, which have little or no cytotoxic effect when applied on MCF 10A cells alone, showed a significant and strong cytotoxicity at all doses and hours when administered in combination. This also underlines the synergistic effect of these two compounds. In other words, although when applied individually, PS showed a dramatic hormetic effect on MCF-10A, PB showed a milder hormetic effect. On the other hand, the combination of PS and PB induced a high cytotoxic effect, which indicates a synergistic effect of these substances on MCF-10A. On MCF-7, only PS was cytotoxic among these two, yet together they show cytotoxic effect on later intervals.

PS and PC combination demonstrated the hormetic effect on the 24th h at low doses on MCF-10A. The severe proliferative effect of PS at early doses seems to be inhibited when applied together with PC. Their combination showed dose dependent cytotoxicity on MCF-7. Generally, in the case of the MCF-7 cell line, in all two-substance combinations, dose dependent cytotoxic effects were observed rather at later time periods; no significant toxicity was observed at the 24th h, and effects were more prominent at the 72nd h. On the other hand, PS-PC combination had at all hours cytotoxic effects on MCF-10A. Therefore, when two-substance combinations are taken into account, they were all toxic on MCF-10A, which means that they do not have a selective anti-carcinogenic effect.

The mixture containing all these flavonoids showed cytotoxicity on MCF-10A at higher doses, however, on both MCF-7 and MDA-MB-231 a cytotoxic effect was observed with 50% doses at all 3 h, which is preferable in terms of cancer treatment. In all substance treatments, Annexin V analyses confirmed the cytotoxic effects as apoptotic.

In conclusion, in this study in which the individual, dual or triple combined effects of PB, PS and PC flavonoids were evaluated, we demonstrated that pinobanksin and pinocembrin had a significant synergistic effect in breast cancer cell lines, as well as the possible hormetic effect of PB on MCF10A. Our results show how important the presence of these substances in combination and different levels, which are present in large amounts in poplar-type propolis. It is clearly demonstrated that different combinations may have different cytotoxic effects, sometimes even proliferative effects. It has been observed that when a compound that is proliferative alone in lower doses when administered in combination may show inhibitory effects in the cell and impairs its effect. In addition, some combinations seem to increase their own effect by showing synergistic effects. Therefore, these studies emphasize the importance and need of analyzing the content and standardizing propolis as a natural treatment approach.

Availability of Data and Materials

The datasets generated during and analyzed during the current

study are available from the corresponding author (O. Öztürk) on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Conception/Design of Study- O.O., A.M.; Data Acquisition- A.B.C., A.P.E.; A.M.; Data Analysis/Interpretation- A.B.C., F.P., T.O., O.O.; Drafting Manuscript- A.B.C., A.P.E.; Critical Revision of Manuscript- T.O., H.Y.A.; Final Approval and Accountability- O.O., A.M.

Informed Consent

Not applicable.

Ethical approval

The study does not require ethics committee approval.

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Research Article

Comparison of Some Balancing Methods for Classification of Pacing Horses Using Tree-based Machine Learning Algorithms

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ABSTRACT

Classifiers in machine learning work on the principle that the observations are evenly distributed across the classes. However, real-world datasets frequently exhibit skewed distributions of classes, which is called imbalanced, causing the classifiers make highly biased predictions. One of the several method groups that deal with imbalance data problem is class balancing methods. We aimed to compare some class balancing methods during the classification of pacing horses according to their origins. Data set contains morphological traits of horses and four origin classes with different sample sizes that leads a multi-class imbalanced data problem. Training data set was modified with different balancing methods. Each balanced data set was trained with C5.0, Random Forest and Extreme Gradient Boosting Machine classifiers. Method comparisons were made based on comparison metrics using the original test set. The best prediction result was obtained on the data set balanced with random undersampling method regarding both G-mean and Matthews Correlation Coefficient; however, the best result according to F1 score was observed on the data set balanced with Adaptive Synthetic Sampling Approach (ADASYN). Primary important variables of the best models were body length, withers height, chest circumference and rump height. The Bulgarian origin was the most accurately predicted class despite having the smallest sample size. Class balancing methods clearly improved the performance of classifiers for predicting origins of pacing horses.

Keywords: Class balancing methods, Imbalanced data, Machine learning, Multi-class classification, Pacing horses

Introduction

Classification is a supervised learning technique in machine learning, where the model attempts to predict a proper label or class for a given data. Recently, classification algorithms or classifiers have been employed in veterinary medicine [1-4], as well as in areas such as disease identification [5,6] and fraud detection [7].

Many classifiers provide high prediction performance when they work with a balanced data set, where the numbers of observations are almost equal in each class. However, real world data sets are commonly *imbalanced*, in which one class could be represented by a lot of observations called *majority class*, while the others are only represented by a few called *minority class* [8]. Many studies proposed some rules such as *imbalance ratio* to define a data set as *imbalanced*. The imbalanced ratio (IR) is defined as the ratio of number of observations of the majority class to minority class [9]. Although several IR values have been proposed, there is still not a clear rule [10]. For instance, in Fernandez et al.'s study [11], datasets with an IR greater than 1.5 are regarded as imbalanced.

According to several studies, classifiers can perform well on imbalanced data sets with clear class separation, since the main idea behind classifiers is to find optimum decision boundaries [12,13]. The actual issue that causes classifiers to struggle with imbalanced data sets reveals as overlapping regions. Overlapping regions occur when data points from various classes are relatively near to each other or when class boundaries overlap. These regions have an adverse impact on the classification task, since they reduce the representative power of the minority classes with small sample sizes [13,14].

In order to effectively deal with multiclass imbalance problem, some approaches are proposed and grouped

under three main titles, (i) data preprocessing methods, (ii) inbuilt mechanisms and (iii) cost sensitive methods. Data preprocessing methods also named as class balancing methods involve modifying the original dataset to create a more balanced class distribution [11,13].

Pacing horses has a popular place in Türkiye. While they were once used for transportation, they now mostly compete in races [15,16]. Despite the fact that Turkish Native breeds are frequently seen in the field, new origins including Iranian, Afghan, and Bulgarian horses have lately been introduced and have found a place among the other pacing horses. Apart from their pacing, the common or distinguishing traits of these horses are still limited and were only subjected to morphological comparison with classical approaches by Yüceer et al.[16] and Çağlayan et al.[15] so far. Therefore, identifying the differences and classifying these origins correctly created a new field of study. Accurate classification according to origin is essential for breed preservation, breed management and genetic improvement. It may also contribute to the cultural heritage and traditions associated with these horses.

This paper attempted to address the classification challenges associated with the origin of pacing horses using morphological traits, and the effectiveness of different balancing methods in improving classification accuracy. In this context, the purpose of this study was (i) to compare and evaluate some class balancing methods during classifying the origins of pacing horses, (ii) to examine the predictive performance of tree-based classifiers, (iii) to assess the relative variable importance values of the best predictive classifiers, and (iv) to draw the attention of those who encounter the problem of class imbalance in the field of veterinary medicine and to offer a solution.

Material and Methods

Data Set

The data set used in this study consists of 430 pacing horses raised in different geographical regions of Türkiye and aged 4 years or older. The class variable is the origin of pacing horses, namely Iranian, Afghan, Bulgarian and Turkish Native. Classifiers are trained on morphological traits such as body length, cannon bone circumference, chest circumference, chest depth, head length, rump height and withers height. Detailed summary statistics are given in *Table 1*. Turkish Native origin that has the highest number of observations is the majority class, while the others are minority classes. A multi-class imbalanced situation is indicated by the IR values, which are 4.79 for Iranian, 10.53 for Afghan, and 17.56 for Bulgarian origin.

Class Balancing Methods

Class balancing methods have the advantage of being more adaptable because their use is independent of the classifier chosen [11]. Many class balancing methods are proposed in previous studies that concentrate on modifying the training data to build an effective classifier. In terms of balancing data sets, we can differentiate between methods that create new observations for minority classes are called oversampling and those that eliminate observations from the majority class are called undersampling. Some combinations of these methods are also commonly used.

Table 1. *Summary statistics of morphological traits [Mean ± Standard deviation; Median (Minimum-Maximum)]*

Morphological Traits	Origin			
	Iranian (n=66)	**Afghan (n=30)**	**Bulgarian (n=18)**	**Turkish Native (n=316)**
Body length	150.25±4.44	148.33±4.6	165.78±5.98	145.49±5.78
	150 (141-163)	148 (142-160)	167 (156-173)	145 (131-164)
Cannon bone circumference	17.63±0.68	17.57±0.57	19.28±0.73	17.07±0.88
	17.5 (15.5-19.5)	17.5 (16.5-18.5)	19 (18.5-21)	17 (14-20)
Chest circumference	159.12±4.64	157.47±4.1	179.11±9.34	156.44±7.15
	159 (148-175)	156.5 (153-167)	180.5 (162-200)	156 (137-174)
Chest depth	63.83±2.11	63.6±2.71	70.44±2.45	61.92±3.31
	64 (58-68)	63.5 (58-68)	71 (65-75)	62 (51-77)
Head length	53.41±1.7	53.05±1.32	57.28±2.02	52.54±1.63
	53 (50-57)	53 (51-55)	57 (53-61)	53 (47-57)
Rump height	144.24±3.43	142.68±3.44	157.56±4.05	139.67±4.67
	144 (136-153)	142 (138-152)	159 (151-163)	140 (127-151)
Withers height	143.65±3.14	142.97±2.52	156.39±3.76	138.92±4.87
	143 (135-151)	143 (139-149)	156 (147-162)	139 (123-151)

In this paper, nine different class balancing methods were used, which are determined based on their superior results in previous studies [12,17]. Random oversampling (ROS), Synthetic Minority Oversampling Technique (SMOTE), and Adaptive Synthetic Sampling Approach (ADASYN) were oversampling methods while Random undersampling (RUS), Tomek links (TL), One-sided selection (OSS), and Edited nearest neighbor (ENN) were used as undersampling methods. SMOTE+TL and SMOTE+ENN were used as combination methods. Although balancing methods are initially developed for binary classification, their application has expanded to multiclass scenarios with pairwise class implementations. Methods are briefly described below.

ROS: This is a non-heuristic method that replicates minority class observations randomly to balance the classes. Though it is easy to implement, it may increase the overfitting [11,14].

SMOTE: It is an oversampling method that contributes new observations to the minority classes without replicating. SMOTE uses interpolation technique that is a type of estimation, where new data points are created within the range of known data points [18]. As a result, with using SMOTE the overfitting issue is avoided. However, it could result in the minority class's decision boundaries expanding into the space of the majority class [12].

ADASYN: Unlike the SMOTE method, it gives weight to minority class observations that are difficult to classify and uses less of those that can be classified easily. It performs the interpolation with the minority class observations and the nearest minority or majority class neighbor observations [19].

RUS: This is also a non-heuristic method that removes majority class observations randomly to balance the classes. Disadvantage of this method is that it can eliminate potentially useful observations and leads to information loss [11,12,14].

TL: Let two observations be e_i and e_j belonging to different classes with the distance $d(e_i, e_j)$ between them. A pair (e_i, e_j) is called TL, if there is no example e_l, such that $d(e_i,e_l) < d(e_i, e_j)$ or $d(e_j,e_l) < d(e_i, e_j)$. TL can be applied as an undersampling method or as a data cleaning method. As an undersampling method, only observations of the majority class are eliminated, and as a data cleaning method, examples of both classes are removed [20].

OSS: This is a two-stage undersampling method. After the results obtained from the application of TL, Condensed Nearest Neighbor (CNN) rule is applied on the observations. TL is used as undersampling method to remove the noisy and borderline observations of majority class. CNN aims to remove examples from the majority class that are distant from the decision border [21].

ENN: It is an undersampling method that uses k nearest neighbor method, where k is equal to 3. This method eliminates a majority class observation unless there are more majority class observations among its three nearest neighbors [22].

SMOTE+TL: Creating synthetic observations with SMOTE can make minority class observations to expand too close to the majority class space. To create better-defined class observations, TL is applied to the over-sampled data set as a data cleaning method. Thus, not only majority class observations, but also minority class observations are removed [12].

SMOTE+ENN: The idea of this method is similar with SMOTE+TL. First SMOTE is applied on data set to create synthetic observations for minority class. Then ENN is applied as a data cleaning method by removing observations from both majority and minority classes [12].

Classification Methods Used in the Study

Tree-based algorithms were used in this study. C5.0 was preferred as single tree. Extreme Gradient Boosting Machine (XGBM) and Random Forest (RF) were regarded as tree-based ensemble learning methods, where XGBM belongs to the boosting family, and RF to the bagging family. The remaining part of this section provides a brief description of the classifiers chosen for our study and how they are applied to the dataset.

C5.0 is s single tree that is an extent work of C4.5 decision tree [23]. It provides high accuracy by using boosting technique. C5.0 has strong opinions about pruning and handles a lot of the choices automatically using defaults that are generally acceptable.

RF is a commonly used ensemble learning method that combines various decision trees to produce a single outcome. Diversity of the trees in the RF is based on two characteristics: bootstrapping the original training data and selection of a random subset of the variables at each split during tree building. Final outcome of the RF is decided based on averaging or majority voting of the trees for regression and classification, respectively [24,25].

XGBM is a more accurate, fast and scalable implementation of gradient boosting decision trees (GBDT) that train an ensemble of decision trees iteratively with boosting technique. The concept behind gradient boosting is to use gradient descent algorithm over an object function to combine a single weak classifier with other weak classifiers to build a strong classifier. In this process, it is aimed to minimize the prediction error considerably [26].

The existence of multiple classes implies an extra challenge for machine learning algorithms because the boundaries of the classes may overlap that leads poor performance.

Class binarization techniques are used to convert the initial multiple-class problem into binary subsets that are simpler to distinguish. In this study, multi-class issue is divided into more straightforward binary classification tasks with one-versus-all approach. Solutions are created to deal with two-class imbalanced datasets for each binary classification task [11].

In this study, original data set is randomly divided into the training and test set with 70% and 30 % ratio, respectively. Training set is used to train the models, while test set is used for testing the examined model for performance comparison purposes. Before building the models, class balancing methods are applied on the original training set. Test set is retained original, while different balanced training sets are created. Although there are many settings that can be used with each machine learning algorithm, we choose the best configuration based on parameter tuning, which offers the parameter set with the best prediction on train sets. Parameter tunning is carried out with applying 10 times repeated 10- fold cross validation technique on the training set. In each repeat, 10-fold cross-validation is applied by splitting the training data 10 equal folds. Each fold is used as validation set for the trained model where the remaining folds are used as training. In addition to determining the best class balancing method, relative variable importance values of the classifiers offering the highest performance are also given. These values present which variables significantly impact the model performance and its predictions [25,27,28].

All calculations in this study are performed with R version 4.2.2 [29] using R Studio (version: 2022.07.2+576) [30]. The R packages Caret (Classification and Regression Training) [28] and UBL (Utility-Based Learning) [31] are used for training the models for each examined classifier and balancing training data sets, respectively.

Model Comparison Metrics

Although various metrics have been proposed over time to compare the performance of classifiers, not all of them are suitable for use in the case of class imbalance. Some metrics such as accuracy or error rate are biased in favor of majority classes [9,32]. The most frequently used metrics in imbalanced data problems are precision, recall, F1 score, G-mean, and Matthews Correlation Coefficient (MCC) [13,32].

The comparison metrics are computed from a confusion matrix that has two dimensions. One dimension is represented by the actual class of an observation and the other by the class that the classifier predicts. The metrics for binary classification are calculated for each class with using a 2x2 confusion matrix. As a result, names of the classes are changed to *positive* and *negative* class, with positive class denoting the class of interest and negative class denoting the other class. Precision measures the correctly classified positive class. Recall presents the proportion of correctly predicted of all actual positive observations. F1 score is a harmonic mean of precision and recall [33]. Calculation of F1 score is given in (1).

$$F1\ score = \frac{2*Precision*\ Recall}{Precison+Recall} \qquad (1)$$

Precision, recall and F1 score can be expanded to multi-class scenarios by using micro-averaging or macro-averaging methods, which provides a single output for all classes [32]. In this study, macro averaging method was used by weighting the metrics with the number of observations in the classes, that was suggested for multi-class imbalanced scenarios [34].

G-mean is a comparison metric that is calculated by taking geometric mean of the recall values of the classes. An approach introduced by Sun et al.[35] for multi-class scenarios is used in this study. The formula is presented in (2), where c denotes the number of classes.

$$G-mean = \left(\prod_{i=1}^{C} Recall_i\right)^{\frac{1}{C}} \qquad (2)$$

MCC is proposed by Halimu et al.[36] for binary classification problems. It provides a correlation coefficient between actual and predicted observations. MCC also can be expanded to multi-class scenario as MMCC with combining pairwise MCC values of the classes, which is given in (3).

$$MMCC = \frac{2}{C(C-1)} \sum_{i<j} MCC_{(i,j)} \qquad (3)$$

When the calculations of the metrics are examined, if the results of all observations are collected in the negative class, it is possible for F1 score and MMCC to get results that go to infinity [32].

Results

Number of observations for each class in the original and balancing methods applied training sets were given in *Table 2*. Applying oversampling methods made a great increase in the numbers of observations of minority classes. With the use of ROS or RUS, all classes had the same number of observations. ENN made a greater decrease in the number of observations of Turkish Native class with respect to TL and OSS. The use of combined methods resulted in an increase in the number of observations in minority classes and a decrease in the number of observations in the majority class Turkish Native.

The performance results of the classifiers for each balancing method were given in *Table 3*. A non-computable issue was indicated by the label NaN. This situation was met when all observations are collected in one class.

The results of the original data set, where the classes are highly skewed, indicated that C5.0 was the best classifier

Table 2. *Number of observations for each class in the original and balanced training sets*

Category	Balancing Method	Origin			
		Iranian	Afghan	Bulgarian	Turkish Native
Original		47	21	13	222
Oversampling Methods	ROS	222	222	222	222
	ADASYN	226	216	222	222
	SMOTE	211	220	221	222
Undersampling methods	RUS	13	13	13	13
	TL	47	21	13	202
	OSS	47	21	13	197
	ENN	47	21	13	185
Combined methods	SMOTE+TL	205	219	221	215
	SMOTE+ENN	195	208	220	146

among the others with the highest F1 score, G-mean and MMCC values. When the results of the oversampling methods were examined, highest F1 score and MMCC values were observed on the data set balanced with ADASYN, where SMOTE provided the highest G-mean. Classifiers yielded the lowest metric results on the data set balanced with ROS among the oversampling methods. XGBM was the most successful classifier on the data sets balanced with ROS and ADASYN, while RF performed best on the dataset balanced with SMOTE.

As the results of undersampling methods were evaluated, the highest G-mean and MMCC values were obtained on the dataset balanced with RUS, while the highest F1 score was provided on the data set balanced with OSS. C5.0 demonstrated the best performance on the data sets balanced with TL, OSS, and ENN, where tree-based ensemble learning methods were failed.

In consideration with the combined balancing methods, Classifiers outperformed on the data set balanced with SMOTE+ENN than the data set balanced with SMOTE+TL, according to the results of G-mean and MMCC. On the contrary, F1 score indicated that SMOTE+TL was the better combined method.

When the whole class balancing methods were evaluated, the highest values of G-mean and MMCC were observed on the data set balanced with RUS and trained with RF and C5.0, respectively. However according the F1 score, the best performance was observed on the data set balanced with ADASYN and trained using XGBM. Furthermore, the lowest value of F1 score were observed on the data set balanced with RUS. Unlike F1 score, the lowest values of G-mean and MMCC were obtained on the data set balanced with ROS. The precision values of all classes varied between 0.66863 and 0.796, whereas the recall values varied between 0.56696 and 0.80311 *(Table 3)*. It was demonstrated that F1 score and MMCC were directly affected with zero values in the confusion matrix, took values between 0.63872 and 0.76816, and 0.42429 and 0.63915, respectively.

Variable importance values were presented in *Fig. 1* to assess the contribution of each morphological trait in the best predictive classifiers according to the highest values of G-mean, MMCC and F1 score, respectively. Body length, withers height, chest circumference and rump height were seemed to be the common primary used predictors during the training classifiers.

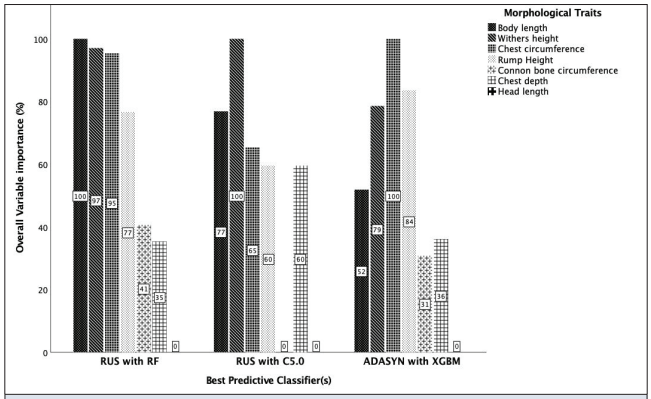

Fig 1. Variable importance values of the best predictive classifiers according to G-mean, MMCC and F1 score

Fig. 2 shows the distributions of the classes corresponding to the original data and the data sets balanced with RUS and ADASYN using the two most significant predictors provided in *Fig 1*. It was found that Turkish Native horses had lower values whereas Bulgarian horses had greater values. Iranian and Afghan horses fell between aforementioned classes. Values of Turkish Native horses seemed to be overlapping on the values of Iranian and Afghan horses on the original training set *(Fig. 2-a)*. As the three graphs were compared, it was seen that the influence of the Turkish Native class on the Iranian and Afghan classes had decreased in *Fig. 2-b*, on which a simpler

Table 3. *Comparison metric results of models for original data and data sets with balancing methods applied*

Category	Balancing Method		Precision	Recall	F1 Score	G-Mean	MMCC
Original		C5.0	0.70322	0.72438	0.70583	0.41825	0.56523
		RF	0.68360	0.65112	NaN	0	NaN
		XBGM	NaN	0.78741	NaN	0	NaN
Oversampling Methods	ROS	C5.0	0.69634	0.66139	0.67738	0.36835	0.42429
		RF	0.67892	0.69289	0.68376	0.34845	0.47818
		XBGM	0.72840	0.66143	0.68970	0.43202	0.54117
	ADASYN	C5.0	0.68321	0.67716	0.67918	0.41039	0.51255
		RF	0.72275	0.74017	NaN	0	NaN
		XBGM	0.76072	0.77951	**0.76816**	0.46568	0.63222
	SMOTE	C5.0	0.74257	0.66143	0.69380	0.49690	0.52644
		RF	0.73781	0.68504	0.70706	0.50214	0.53024
		XBGM	0.66863	0.66928	NaN	0.00000	NaN
Undersampling methods	RUS	C5.0	0.79369	0.56696	0.63872	0.60548	**0.63915**
		RF	0.78624	0.66143	0.70237	**0.65315**	0.63306
		XBGM	0.79600	0.61417	0.67712	0.57847	0.57826
	TL	C5.0	0.72254	0.74799	0.72932	0.46557	0.61450
		RF	0.68316	0.74804	NaN	0	NaN
		XBGM	NaN	0.80311	NaN	0	NaN
	OSS	C5.0	0.73611	0.74802	0.73658	0.49880	0.63213
		RF	0.69704	0.74802	NaN	0	NaN
		XBGM	NaN	0.79527	NaN	0	NaN
	ENN	C5.0	0.72802	0.70079	0.71304	0.50543	0.60556
		RF	0.72542	0.73774	NaN	0	NaN
		XBGM	0.72860	0.77164	NaN	0	NaN
Combined methods	SMOTE+TL	C5.0	0.71800	0.66931	0.68935	0.42070	0.46455
		RF	0.73637	0.70076	0.71647	0.45115	0.56610
		XBGM	0.69039	0.70077	NaN	0	NaN
	SMOTE+ENN	C5.0	0.77362	0.62204	0.67517	0.44493	0.46114
		RF	0.78699	0.64564	0.69118	0.59955	0.63179
		XBGM	0.73502	0.68503	0.70475	0.45843	0.58852

NaN: *Not a Number;* ***ROS:*** *Random Over-Sampling;* ***ADASYN:*** *Adaptive Synthetic Sampling Approach;* ***SMOTE:*** *Synthetic Minority Oversampling Technique;* ***RUS:*** *Random Under-Sampling;* ***TL:*** *Tomek Links;* ***OSS:*** *One sided selection;* ***ENN:*** *Edited Nearest Neighbor;* ***RF:*** *Random Forest;* ***XGBM:*** *Extreme Gradient Boosting Machine;* ***MMCC:*** *Multi-class Matthews Correlation Coefficient. Bold values present the highest comparison metric in a column*

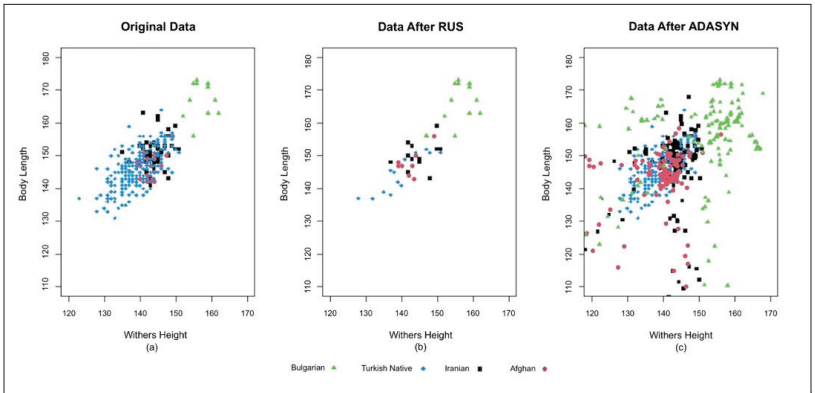

Fig 2. Distributions of the classes belong to the original data, data balanced with random undersampling, and data balanced with adaptive synthetic sampling approach

RUS: Random Under-Sampling; ADASYN: Adaptive Synthetic Sampling Approach

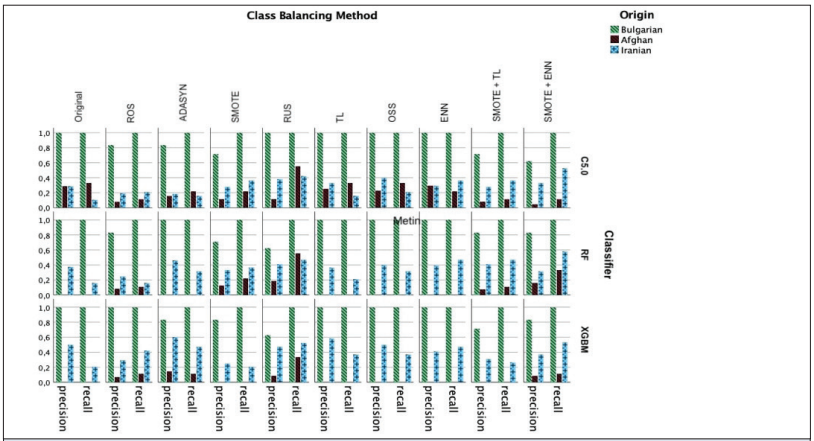

Fig 3. Precision and recall values of minority classes for each balancing method and classifier. *(ROS: Random Over-Sampling; ADASYN: Adaptive Synthetic Sampling Approach; SMOTE: Synthetic Minority Oversampling Technique; RUS: Random Under-Sampling; TL: Tomek Links; OSS: One sided selection; ENN: Edited Nearest Neighbor; RF: Random Forest; XGBM: Extreme Gradient Boosting Machine)*

distribution of the classes can be obtained. The highest number of observations were seen in *Fig. 2-c*, where the distribution of the classes seemed to be more complicated.

Fig. 3 displays the precision and recall results of the minority classes for each class balancing method and classifier. All classifiers that were trained on different balanced data sets had superior prediction performance on Bulgarian horses. It was followed by Iranian and Afghan horses, respectively. Classifiers presented quite poor prediction performance on Afghan horses. Precision and recall values of Afghan horses were found to be quite related to the non- computable results in *Table 3*.

Discussion

In this study we compared some well-known class balancing methods and tried to improve performances of classifiers on classification of pacing horses according to their origins in Türkiye. Class balancing methods, that were categorized under three categories, applied on the training data sets before training the classifiers. Classifiers provided the most successful prediction performance on the data set balanced with RUS according to G-mean and MMCC metrics, where F1 score indicated that best predictive performance was on the data set balanced with ADASYN. Using RUS and ADASYN increased the predictive performance of the classifiers with respect to original data set. Surprisingly, RUS, which was usually regarded as an inefficient method, produced comparable results to more complex methods. This result was consistent with some previous studies. In their study, Drummond and Holte [37] draw some conclusions about how balancing methods enhance the performance of the C4.5 algorithm. They compared over and under-sampling methods using cost curves and found that under-sampling methods produce better results. Ling and Li [38] also compared over and under-sampling for boosted C4.5 and conclude that under-sampling provides superior results, though over-sampling produces nearly as well. On the other hand, there are some studies in the literature that favor oversampling methods. On several data sets, Batista et al.[12] compared the effects of over-sampling, under-sampling, and combined methods. They concluded that over-sampling methods generally outperform the competition. They reported that over-sampling methods outperformed than the other ones in general. Even they proposed some combined methods such as SMOTE+ENN and SMOTE+TL to the literature, concluding that random-oversampling methods provided more meaningful results. Japkowicz and Stephen [14] made a systematic study on class imbalance study with using artificial data sets. They compare various over-sampling and under-sampling methods and conclude that over-sampling had a better way to reduce error rate.

In this study, we also compared some tree-based classifiers. Although RF provided the best prediction performance with respect to G-mean or XGBM provided the highest F1 score, it should be noted that C5.0 algorithm did not produce any NaN results *(Table 3)*. Therefore, we can call C5.0 as the best classifier in the study since it worked well in every scenario. The performance of the classifiers had been negatively impacted by the fact that Afghan horses were not only a minority class but also had a data set that overlaps with Iranian and Turkish Native horses *(Fig. 2-a)*. In the study, although the Bulgarian horses were relatively few and contributed little to the formation of the classifiers, the predictability rate was higher in all scenarios. This supports the idea that in a classification problem with an imbalanced data, having clear decision boundaries can overcome the issue of working with different sample sizes [9,12].

There is one undersampling and one oversampling method that is observed to be the most successful in improving

the prediction performance in the classification problem performed with the data set used in this study, since the highest comparison metrics were observed on the data sets balanced with these methods. While the highest G-mean and MMCC values were observed with the RUS-balanced data set, the highest F1 score value was observed on the data set balanced with ADASYN. On the contrary, the lowest G-mean and MMCC values were seen in the data set balanced with ROS, while the lowest F1 score was seen on the data set balanced with RUS. While G-mean and MMCC were in agreement, F1 score provided results in the opposite direction. In some studies, containing binary or multiclass imbalanced data problems using MCC and MMCC over F1 score were highly recommended. They concluded that F1 score might lead some biased results on imbalanced data scenarios [32,39].

Following the selection of the best-balanced data set and classifier combination, the morphological traits that had contributed the most to the training of the classifiers were evaluated. Body length, withers height, chest circumference, and rump height were determined as predictors that had major roles in classifying pacing horses *(Fig. 1)*. They were followed by chest depth, cannon bone circumference and head length. When the distribution of the classes drawn with body length and withers height were examined *(Fig. 2-a)*, it was observed that Turkish Native class had the lowest measurements, while the Bulgarian horses had the largest. Iranian and Afghan horses, that present overlapping, obviously had similar morphological traits which was supported by Yüceer et al.[16]'s study.

This study is important as it applies machine learning techniques, which are rising in popularity, to the practice of veterinary medicine. This work is useful for several reasons, including the comparison of methods that can be used to solve the imbalance problem across classes when using classification-based machine learning algorithms, as well as the possibility that it will serve as a basis for further research. Also, the Turkish Ministry of Agriculture and Forestry has prioritized breed registration studies in recent years. There are numerous registered breeds among animals other than horses. For the registration of horse breeds, a scientific data and a pedigree are required. In this study, morphological traits were used to classify the pacing horses in Türkiye, including native, Iranian, Afghan, and Bulgarian horses. As a result, the phenotypic diversity of the pacing horses raised in various Turkish regions was identified, and it was found that body length, withers height, chest circumference, and rump height were seemed to be the primary used predictors during training the classifiers. Consequently, this study generated crucial data that can be applied to research on breed registration. Additionally, it might be used for a second-order validation task.

In conclusion, loss of prediction accuracy in a multi-class problem is related to both the presence of numerous minority classes and situations of class overlapping. Class balancing methods can be used to overcome these issues, which were also present in our data set. In comparison to the original imbalanced data set, superior prediction performance results were obtained on the data sets balanced with RUS and ADASYN. Future research may investigate various methods that address the issue of imbalanced data to enhance the classification of pacing horses.

Availability of Data and Materials

The dataset used in the study is available from the corresponding author (H. Özen) on reasonable request.

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This study was not financially supported.

Competing Interests

The authors have no conflicts of interest to declare.

Ethical Approval

There is no need for ethical committee approval in the dataset used for this study given that no interventional procedure was involved and only data on morphological traits were used.

Author Contributions

H.Ö. and D.Ö. designed the study and made literature research. H.Ö. and D.Ö. did methodological work and wrote the paper. B.Y.Ö. and C.Ö collected the data set and made contributions to results and discussion.

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Research Article

Detection of *Babesia bigemina* Cases in Dogs in Rural Areas/Türkiye by Molecular Methods

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Abstract

Babesiosis, a very important vector disease, has also been detected in humans and animals. This study was carried out on 120 whole blood samples taken from Cankiri Municipality Animal Care and Rehabilitation Center (Türkiye) between December 2021 and 2022 in Cankiri. In this study, venous blood samples taken by a veterinarian were used for diagnosis. The 18S rRNA gene region was examined with conventional PCR analysis and the positive results were subjected to sequence analysis. As a result of sequence analysis, these positive cases were determined to be *Babesia bigemina***.** Additionally, as a result of statistical analysis, it was found that dogs detected positive for *B. bigemina* did not give significant results according to age, gender or symptoms. *B. bigemina* detected in this study were registered in the NCBI database with 7 (Acc no: OQ186727), 12 (Acc no: OQ186720), 19 (Acc no: OQ186721), 21 (Acc no: OQ186722), 23 (Acc no: OQ18723), 28 (Acc no: OQ186724), 30 (Acc no: OQ186725), and 70 (Acc no: OQ186726) numbers. The *B. bigemina* species detected in dogs in this study is actually seen in farm animals. This study is the first detection report of *B. bigemina* in dogs in Turkey. As a result, *Babesia* species seen in dogs need to be reconsidered and investigated with the development of molecular techniques.

Keywords: *Babesia, Babesia bigemina*, Babesiosis, Dog

Introduction

Babesiosis is a tick-borne zoonotic disease caused by the *Babesia* protozoan parasite that infects red blood cells. Babesiosis, which is a very important vector disease in terms of public health, has also been detected in many different animal species, including humans. Transmission by ticks often occurs in tropical and subtropical regions during the warmer months [1].

Babesiosis is a disease described first in cattle [2,3]. In Turkiye, babesiosis cases were detected in cattle in 1969 [4]. Babesiosis in dogs were also reported in Europe [5]. *Babesia* spp. infection can be observed in dogs with lethargy, anorexia, fever, jaundice, hemolytic anemia, hemoglobinuria or bilirubinuria, weight loss, and death [6,7].

According to their size, *Babesia* species are divided freely into two groups: small *Babesias* containing *B. microti, B. gibsoni*, etc., and large *Babesias* containing *B. bovis, B. canis*, etc. Only the trophozoites of *B. divergens* are small and are in the same group as the larger *Babesias* [8]. The causative agents of canine babesiosis are the larger *Babesias* (3-5 µm in size), *B. canis* and the smaller (1-3 µm in size) *B. gibsoni* [8]. With the increase in molecular studies, *B. canis* was thought to have three subspecies: *B. canis rossi, B. canis canis,* and *B. canis vogeli*. However, it is thought that they may be in a different group due to important differences in clinical, geographical distribution, and vector specificities.

In addition, three small *Babesia* species, such as *B. gibsoni, B. conradae,* and *B. microti,* are known to infect dogs. Unlike *Babesias* encountered in dogs, a large-formed *Babesia* species related to *B. bigemina* has been reported from North Carolina in the United States [10-12].

This study aims to detect the 18S rRNA gene for *Babesias* in blood samples of stray dogs in rural areas of Türkiye and to perform sequence analysis of those found positive.

Material and Methods

Ethical Statement

This study was approved by the Local Ethics Committee of the Veterinary Control Center Research Institute (Etlik/Ankara) (Approval no: 2021/23).

Sample Collection

This study was carried out under veterinary supervision on 120 whole blood samples taken from Cankiri Municipality Animal Care and Rehabilitation Center between December 2021 and 2022. The blood collected was taken into 5 mL tubes from the venous blood of the dogs coming to this center under the supervision of a veterinarian and used for the diagnosis of the animals. The demographic information of the dogs was also recorded by the veterinarian.

Molecular Analysis

DNA extraction: A commercial kit (Gene MATRIX series, Quick Blood Lot No. F/280921) advised extracting 120 EDTA blood samples from dogs using a spin column. The obtained DNA was stored at -20ºC until the conventional PCR study.

Primer design for detection of DNA and conventional PCR: Using forward and reverse primers, the 18S rRNA-specific gene region was aimed in standard PCR. The 18S rRNA primers were made in Turkiye at BM laboratories. The 18S rRNA primers we used in conventional PCR analysis are capable of detecting *Hepatozoon* spp., *Babesia* spp., *Theleria* spp., and *Hemovilia mauritacana*. PCR mix, for a total of 45 µL for each sample: Distilled water is 33.75 µL, 10 x PCR buffer is 5 µL, Mg Cl2 (25 mM) is 3 µL, d NTP mix (10 mM) is 1 µL, Primer I (10 pmol) is 1 µL, Primer II (10 pmol) is 1 µL, and Taq DNA polymerase (5 U/µL) is 0,25 µL. For this purpose, BJ1 (5'-GTCTTGTAATTGGAATGATGG-3') and BN2 (5'-AGTTTATGGTTAGGACTACG-3') primers and protocols were used for the amplification of the 18S rRNA gene region [13].

A 45 µL of this master mix was dispensed into the tubes, and 5 µL of sample DNA was added to each tube to bring the total reaction volume to 50 µL. Samples prepared according to this mixture were placed in a thermal cycler for 5 min at 95ºC, 30 sec at 95ºC, 45 sec at 52ºC, 45 sec at 72ºC, and 5 min at 72ºC for reproduction.

After that, PCR products were detected by 2% agarose gel electrophoresis. In this study, 100 bp ladders were used for identification, and fragments falling in the range of 400-500 bp were examined. Suspicious positives falling between 400 and 500 bp in the target region were subjected to sequence analysis. As a result of sequence analysis, *B. bigemina* was detected. The appearance of 400–500 bp regions was considered positive, and DNA sequence analysis was performed on positive samples.

Sequencing and Phylogenetic Analyses

All conventional PCR-positive samples were sequenced in one direction at a commercial sequencing service provider (BM Laboratories, Türkiye). Nucleotide sequences were analyzed using the online nucleotide Blast system (National Center for Biotechnology Information, www.blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analysis was determined using the Maximum Likelihood method and the Tamura-Nei model [14]. When drawing the *B. bigemina* phylogenetic tree, approaches like the reference strains, where the reference strains came from, which countries they were studied in, and how far apart they were from each other were also tested. This analysis involved eight (7, 12, 19, 21, 23, 28, 30, 70) nucleotide sequences. Evolutionary analyses were conducted in MEGA X [15].

Statistical Analysis

Statistical analyses of data were performed using SPSS version 26 software. Descriptive features are given using frequencies and percentages. The difference between the groups in terms of these frequencies was compared using the Chi-square test. A value of $P<0.05$ was accepted as the significance limit.

Results

Demographic data obtained from dogs in this study and positivity rates according to conventional PCR results are given in *Table 1*. The dogs observed in this study were mostly male (51.67%) and one-year-old (56.67%). The most common symptom among the dogs in this study was redness (18.33%), itching (17.5%), and cachexia (9.17%). As a result of statistical analysis, *B. bigemina* positivity rates did not yield significant results according to age, gender, and symptoms ($P>0.001$).

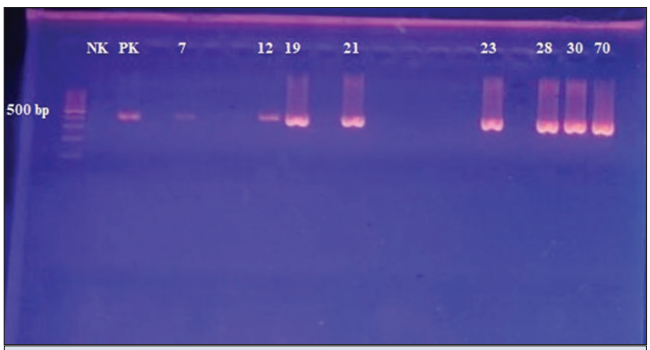

Fig 1. Conventional PCR gel image of *Babesia bigemina* in whole positive samples

Table 1. *Distribution of demographic data according to Babesia bigemina positivity (n=120)*

Demographic Data of Dogs		*Babesia* spp.		Total (%)
		Positive	Negative	
Age	1	5	63	68 (56.67)
	2	2	35	37 (30.83)
	3	0	12	12 (10)
	4	1	2	3 (2.55)
Gender	Male	3	59	62 (51.67)
	Female	5	53	58 (48.33)
Lesion	Alopecia	2	5	7 (5.83)
	Diarrhea	0	1	1 (0.83)
	Cachexia	0	11	11 (9.17)
	Itching	0	21	21 (17.5)
	Itching Alopecia	0	2	2 (1.67)
	Redness	2	20	22 (18.33)
	Redness Alopecia	0	2	2 (1.67)
	Vomiting	0	2	2 (1.67)
	Vomiting diarrhea	0	2	2 (1.67)
	Breaking nails	0	1	1 (0.83)
	None	4	45	49 (40.83)
Total		8 (6.67)	112 (93.33)	120 (100)

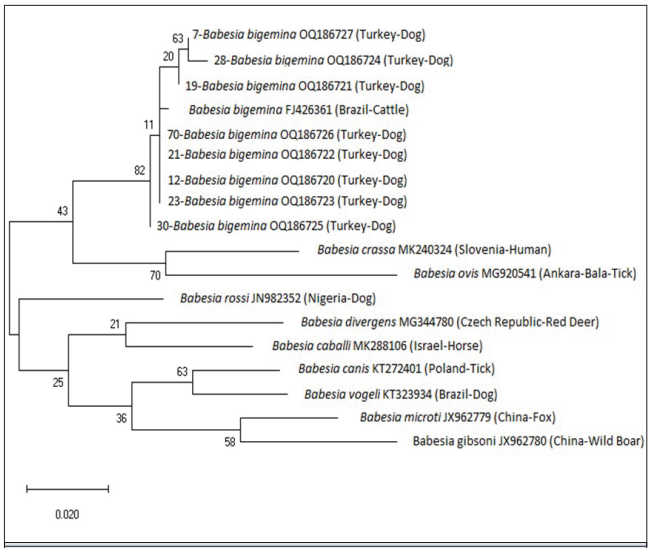

Fig 2. Phylogenetic tree of *Babesia bigemina* specimens found in this study

Of the 120 dogs tested, 8 were positive by conventional PCR *(Fig. 1)*. *B. bigemina* was found in eight of the positive samples (7, 12, 19, 21, 23, 28, 30, 70) in the sequence analysis performed (BM laboratories, Türkiye). These positive data were registered in the NCBI database as 7 (Acc no: OQ186727), 12 (Acc no: OQ186720), 19 (Acc no: OQ186721), 21 (Acc no: OQ186722), 23 (Acc no: OQ18723), 28 (Acc no: OQ186724), 30 (Acc no: OQ186725), and 70 (Acc no: OQ186726) *(https://www.ncbi.nlm.nih.gov/)*. The phylogenetic tree was drawn with reference strains and given in *Fig. 2*. When blasted with the NCBI database, all samples detected positive in dogs were investigated for their relationship with the *Babesia* species detected so far. It was found to be 100% compatible only with *B. bigemina*. The *B. bigemina* species detected in this study are closely related to the *B. bigemina* species, which was also detected in cattle in Brazil. *Babesia* species in this study are in the same clade and in the same branch *(Fig. 2)*.

Discussion

Babesiosis is a protozoan disease. Babesiia species cause hemoglobinuria, and can be infectious in dogs as well as cattle and sheep [16]. Although *Babesia* is an important human problem in the world, it continues to pose a threat to veterinary medicine. This threat is also important in terms of economic losses. Accordingly, this parasite has been poorly studied compared to its malaria-causing relative, *Plasmodium* [17].

Studies on dogs in Türkiye were conducted in Istanbul *(B.canis, B. vogeli, B. rossi)* [18], in Kayseri *(B. canis canis, B. gibsoni, B. canis vogeli)* [19], in Konya *(B. canis vogeli)* [20], in Erzurum *(B. canis)* [21], and in Diyarbakır *(Babesia spp.,*

B. canis, B. vogeli) [22]. *B. canis, B. vogeli, B. rossi,* and *B. gibsoni* are the most common *Babesia* species in dogs in our country and in the world. However, other *Babesia* species can be detected, apart from those frequently seen in dogs. The severity of the disease depends on the type of *Babesia* [6]. The 18S rRNA primers we used in conventional PCR analysis are capable of detecting *Hepatozoon* spp., *Babesia* spp., *Theleria* spp., and *Hemovilia mauritacana*. Suspicious positives detected after sequence analysis were found to be *B. bigemina*. This is the report of *B. bigemina* in dogs from Turkiye. This infective agent was determined to be *B. bigemina* when positive reactions detected were blasted with the NCBI database. A review of previous studies revealed that an unnamed species of *Babesia* closely related to *B. bigemina* has been reported from North Carolina in the United States [10, 23].

As a result, it is possible for dogs to become infected with unexpected *Babesia* species. The emergence of extensive molecular techniques and the rise of rigorous research support this. Thus, *Babesia* infections in dogs gradually began to be clarified. In addition, another study that mentions the presence of *B. bigemina* in dogs shows this. In this study, this agent was detected in canines living on cattle farms [24]. It should be noted that *B. bigemina* bearing ectoparasites can be shared between livestock and wildlife. This may explain the distribution, but the distribution of tick-borne *Babesia* species in dogs is still not fully understood [25].

B. bigemina, one of the larger *Babesia* seen in cattle, is carried *by Boophilus* spp., *R. sanguineus, R. bursa, R. evertsi, R. microplus, R. annulatus, Haemaphysalis* spp., *Amblyomma parvum* [25-29]. Although there is a wide variety of these ticks in Europe, it cannot be assessed that how many of these ticks dogs carry and how many of them have infections. Interestingly, *B. bigemina* infections were detected in dogs in this study. The fact that *B. bigemina* can be carried by many different ticks may indicate that the infection can also be seen in dogs. One of the limitations of this study is that tick samples, if any, were not collected from the dogs that were drawn blood.

It has been reported that merozoites circulating in the blood can be transmitted to a healthy host by direct blood transfusion. In America, Australia, and Europe, *B. gibsoni* has been found to be transmitted vertically and through wounds, (fighting dogs), saliva, or blood draws [2]. This issue also makes us think of other unreported *Babesia* species. In this study, it was determined that dogs with *B. bigemina* were in close contact with each other and became close by fighting.

Babesia species that affect dogs are thought to be of zoonotic importance [30]. However, human babesiosis is a rare disease, and data on the causative protozoan species is quite lacking. Zoonotic babesiosis is caused by *B. divergens* transmitted from cattle and *B. microti* transmitted from rodents. Therefore, it is thought that *B. bigemina* detected in this study is not zoonotic. However, this needs further investigation. Detection of a species originating from cattle is rare in dogs, and its zoonotic character should be investigated [2].

Although molecular methods are expensive and complex, they are preferred because of their high specificity and sensitivity. The use of PCR is becoming increasingly common nowadays. Species identification is also more reliable in PCR [31].

Since the animals investigated in this study were stray dogs, the findings, although clinically apparent, were not distinctive for babesiosis. Typical signs of *Babesia* were not observed in these dogs. Although jaundice is a common symptom of babesiosis infection, it is a rare symptom in dogs [32] and also not detected in this study.

As a result, the *B. bigemina* species detected in dogs in this study is normally seen in farm animals. With this study *B. bigemina* was detected in dogs in Türkiye. *Babesia* species that can infect dogs should be reconsidered with the development of molecular techniques and researches should be increased. By emphasizing the atypical cases encountered, the causes and consequences should be investigated, and the issue should be carefully considered.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author (B. Yucesan).

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Ethical Statement

This study approved by the Local Ethics Committee of the Veterinary Control Center Research Institute (Etlik/Ankara) (Approval no: 2021/23).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

OO and BY planned the study and designed the experiments, OO, BY and YY helped write the article and laboratory process, ZO helped collect samples during fieldwork, and BY, YY helped with data analyses. All authors read and approved the final version of the manuscript.

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RESEARCH ARTICLE

Evaluation of Colostral Passive Immune Transfer Success in Turkish Kangal Shepherd Dogs

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Abstract

The aim of this study was to investigate the passive immune transfer process and its effects in Turkish Kangal shepherd dogs. The material of the study consisted of 15 dams and their 138 offspring in the region of Central Anatolia, Türkiye. Blood samples were collected from the surviving puppies at 48±4 h postpartum and IgG levels were measured using the ELISA method. Before the blood samples were collected, 34 of the puppies (26.64%) died, 56 of the 104 puppies (40.58%) whose blood samples were collected and IgG analyses were done died prior to weaning, and 48 (34.78%) survived until weaning. All of the analyzed blood samples had IgG levels above 230 mg/dL and there was no passive transfer failure. The average blood IgG level of the puppies was 664.86 mg/dL. The effects of litter size and birth season on passive transfer success were statistically significant ($P<0.05$), while maternal age and the sex of the puppy were not significant ($P>0.05$). There was no correlation between the average blood IgG levels of the puppies and group mortality rates ($r = -0.44$). In conclusion, while the rate of passive transfer success for Turkish Kangal shepherd dogs was found to be high compared to other breeds, the high rate of mortality among the puppies suggested that dams and their litters should be cared for more carefully in the neonatal period.

Keywords: Canine neonatal period, Colostrum, ELISA, Ig G, Passive immune transfer, Puppy mortality, Turkish Kangal shepherd dogs

Introduction

Due to their endotheliochorial placental structure, dogs cannot transplacentally transfer macromolecular components such as IgG to their puppies during the intrauterine period [1]. Puppies are born with almost agammaglobulinemia or hypogammaglobulinemia, similarly to calves, foals, piglets, and kittens [2]. The immune components received through colostrum are of great importance in the survival of puppies born with almost completely unformed immune systems [3,4]. This immune transfer from mother to newborn via colostrum is called passive immune transfer [5].

Although many immune components are transferred in passive transfer, the success of this transfer is measured by IgG level [1,6]. While the blood IgG level in adult dogs is 800-2500 mg/dL, it is only 30 mg/dL when puppies are born [1]. Newborn puppies receive 85-95% of the IgG they need from colostrum, reaching an average IgG concentration of 600-1600 mg/dL at 2 days of age [7-9]. Even puppies that experience a successful passive immune transfer process only reach blood IgG levels 50-77% of that of adult dogs until their own Ig begins to be synthesized [1,10].

The critical period of puppies' first 3 weeks of life, before their own Ig levels reach a protective level, is called the neonatal period and the risk of death during this period is very high [11]. Survival in the neonatal period is directly related to the avoidance of passive transfer failure (PTF) [1,12]. The threshold for passive transfer insufficiency in dogs is an IgG concentration of 230 mg/dL and values below that are considered to be indicative of failure. While mortality in the neonatal period remains below 5% in puppies with successful passive transfer processes, it approaches 50% in puppies that experience PTF [10].

Methods used to evaluate passive transfer success in dogs include the determination of blood IgG levels at 2 days of age by ELISA, determination of blood gamma-glutamyl transferase levels, determination of antibody titers against specific diseases such as canine parvovirus 2, and early growth monitoring [1,13-15]. Although the success of passive transfer can be affected by variables such as maternal

age, maternal behavior, litter size, birth weight, and live weight, the quality, quantity, and timing of colostrum consumption are the criteria that determine the success of passive immune transfer [1,10]. It is known that puppy mortality rates among large and giant dog breeds are higher than those of small breeds [16].

Turkish Kangal (Karabaş) shepherd dogs, together with Turkish Akbaş and Kars (Kafkas) shepherd dogs, are world-famous shepherd dogs of Türkiye and are bred in many countries including Belgium, France, Germany, and Slovenia [17,18]. Turkish Kangal shepherd dogs are large-sized dogs like many other breeds of Turkish shepherd dogs [19]. They are among the large breed dogs with an average adult live weight of 50 kg [17,20,21]. Kangal dogs give birth to an average of 6-9 puppies in a litter and the average birth weight of the puppies is 500-550 g [22-24].

This study aims to investigate the passive transfer success of Turkish Kangal shepherd dogs, a breed that is becoming increasingly popular in the world, and the variables affecting the passive transfer process. It is expected that this study will serve as a reference for the investigated markers of large-breed dogs.

Material and Methods

Ethical Statement

The study was carried out according to European Council Directive 2010/63/EU on the protection of animals used for scientific purposes and the relevant Turkish legislation (Law No. 5199, Regulation No. 28141) dated 13.12.2011, Kırıkkale University Animal Experiments Local Ethics Committee approval was obtained before the study (Approval No. 2020(4)/25).

Animals

The animal material of this study consisted of Turkish Kangal shepherd dogs raised as herd protection and guard dogs in the Kırıkkale region. A total of 138 puppies born to 15 dams were followed and 104 puppies that survived for 48 h were included in the passive transfer evaluation.

None of these traditionally fed dogs received professional feeding or dry food. A vegetable protein-based slurry called "yal," made from barley or wheat flour and sometimes with bran added, was used as chow. All of the puppies included in this study were born to dams who had received periodic vaccinations and antiparasitics.

The study was conducted in the 12-month calendar year from December 2020 to November 2021. Kırıkkale province, where the study was conducted, is located at 39.8392°N, 33.5089°E and has a continental climate. The puppies were evaluated within the 4 seasonal groups of winter, spring, summer, and autumn according to their birth dates. In the Kırıkkale region where the research was conducted, winter is December-February, spring is March-May, summer is June-August, and autumn is September-November.

Blood Sampling and IgG Analyses

Direct contact with the puppies was avoided by using disposable protective clothing and examination gloves. The puppies were taken from their mothers by their owners and given to the researchers. After blood sampling, puppies were rubbed on the dam's perineal area by the owner and then attached to the dam's teats to prevent the possibility of the dam rejecting her offspring and ensure that all puppies would be accepted by their mothers after sampling. Blood samples were obtained from the puppies 36-48 h after birth from the cephalic vein by breaking the plastic luer base of a 21-gauge needle. During blood sampling, the puncture area was not shaved. Asepsis and vasodilation were achieved with alcohol and then samples were collected into anticoagulant-free pediatric gel tubes of 1 mL with yellow caps that accelerated clotting with silica particles in the tube walls. Blood samples were centrifuged at 3000 rpm for 10 min, and serum was separated and kept in a freezer at -20°C until analysis was performed. Serum IgG was analyzed using a commercial ELISA kit (Dog IgG ELISA Kit, Lot: E44-128, 210428; Bethyl Laboratories, Montgomery, TX, USA).

Recorded Parameters

Information about the owners of the dams included in the study, puppies' birth dates, dams' ages, breeding purposes, the sex of the puppies, litter size, deaths in the first 48 h, deaths in the first 2 months, and numbers of surviving puppies were recorded. Since there are no previous studies in the literature on the individual marking of newborn Kangal puppies and the reactions of their mothers to such markings could not be predicted, individual marking and tracking of the puppies was not performed considering the possibility of the dams harming their offspring. The offspring of each dam were followed collectively and the IgG values of these groups were obtained by averaging the IgG levels of each individual offspring. Similarly, the mortality rates of the offspring of each dam were recorded, and the mean IgG values and mortality rates for each group were compared at the end of the study.

Informed consent has been obtained for all client-owned animals included in this study.

Statistical Analysis

One-way ANOVA testing was used to determine the differences between the groups in values determined from serum samples obtained in the study. The significance level was accepted as $P<0.05$ and the Tukey test, which is a post hoc test, was used to determine the groups from

which the significant differences between groups arose. The obtained statistical results were presented as mean ± standard error values in tables. The means IgG levels and mortality rates of the groups were evaluated by Pearson correlation test [25].

Results

Within the scope of this study, 138 puppies were born to 15 dams. The mean number of puppies born in litters per dam was calculated as 9.2. Thirty-four (24.64%) of these puppies died within the first 48 h after birth and could not be included in the PTF evaluation because blood samples were not taken. The remaining 104 puppies were included in the PTF evaluation. Of the 104 pups evaluated for PTF, 56 (40.58% of the total puppies) died before weaning (2 months of age) and 48 (34.78% of the total puppies) survived *(Table 1)*.

The IgG levels in blood serum samples obtained from the puppies were found to range between 419.27 and 1965.34 mg/dL with a mean value of 664.86 mg/dL *(Table 2)*. When these blood IgG levels were evaluated, it was determined that all measurements were above the PTF threshold value of 230 mg/dL.

There was no statistical difference (P>0.05) in the Ig levels of the puppies according to maternal age *(Table 2)*.

There was no statistically significant difference (P>0.05) between the Ig levels of the puppies according to sex *(Table 3)*.

There was a statistically significant difference (P<0.05) in the Ig levels of the puppies according to the season of birth *(Table 4)*. This statistically significant difference was observed to arise between puppies born in summer and those born in winter.

There was a statistically significant difference (P<0.05) in the Ig levels of the puppies according to litter size *(Table 5)*. The blood IgG levels of the offspring of dams with litters containing 3-5 offspring were significantly higher than those of offspring in larger litters.

Table 1. *Number of puppies born per litter and puppy losses*

Number of dams in the study	Total number of puppies born	Mean number of puppies in a litter	Number of puppies that died in the first 48 h	Number of puppies that died at 2-60 days	Number of puppies surviving to 60 days of age
15	138	9.2	34 (24.64%)	56 (40.58%)	48 (34.78%)

Table 2. *Comparison of Ig levels of offspring according to maternal age*

Age of dams	Number of offspring (n)	Ig values (mg/dL) of puppies (Mean±S_E)	p
Group I (2 years)	30	654.70±63.32	0.914
Group II (3 years)	38	697.68±56.26	
Group III (4 years)	17	674.28±84.12	
Group IV (5 and above)	19	632.79±79.57	
Total	104	664.86±35.86	

Table 3. *Comparison of Ig levels according to the sex of the puppies*

Groups	Number of offspring (n)	Ig values (mg/dL) of puppies, mean ± standard error	p
Group I (male)	58	667.465±40.75	0.944
Group II (female)	46	672.289±56.56	

Table 4. *Comparison of Ig levels according to birth season of the puppies*

Season	Number of offspring (n)	Ig values (mg/dL) of puppies, mean ± standard error	p
Group I (spring)	21	613.488±46.23	0.046
Group II (summer)	32	554.026±35.26	
Group III (autumn)	36	694.795±56.19	
Group IV (winter)	15	870.236±155.46	

Table 5. *Comparison of serum Ig levels according to litter size*

Groups	Number of offspring (n)	Ig values (mg/dL) of puppies, mean ± standard error	p
Group I (3-5 puppies)	8	1024.127±273.41	0.022
Group II (6-8 puppies)	28	642.336±54.009	
Group III (9-11 puppies)	12	600.910±107.97	
Group IV (12-14 puppies)	56	647.302±31.57	

Table 6. *Correlation of serum IgG levels and mortality rates of the maternal groups*

Parameters	N	Mean group IgG values (mg/dL)	Group mortality percentages	p
Mean group IgG values (mg/dL)	15	1	-0.44	0.877
Group mortality percentages	15	-0.44	1	

The mean IgG levels and mortality rates of the offspring of each dam were calculated. The r value was found to be -0.44 and the P value was found to be 0.877, indicating that there was no correlation between the mean IgG levels and mortality rates *(Table 6)*.

Discussion

Although IgG is not the only immune component present in the colostrum of dogs, it is commonly considered as a reference molecule in the evaluation of passive transfer success [1,9,26]. Gooding and Robinson [27] reported the prevalence of PTF in dogs as 5%. Mila et al.[10], in a study of 149 puppies, found that 26 puppies had IgG concentrations below the threshold value and they subsequently reported the rate of PTF in dogs as 17.4%. Chastant and Mila [1] reported that the IgG concentration was below 230 mg/dL in only 4 puppies among the unpublished data of their evaluations of 90 Labrador puppies; they found the PTF rate to be 4.4%. In the present study, blood samples from 104 puppies were evaluated. It was observed that blood IgG levels were above 230 mg/dL at 48 h of age in all cases and no PTF was observed for any of the evaluated puppies. The results obtained here are similar to those of Gooding and Robinson [27] and Chastant and Mila [1] but considerably lower than the values reported by Mila et al.[10]. Although the rate of PTF was reported to be between 4.4% and 17.4% in other studies of dogs. PTF was not detected in any puppies in the present study. Although the rate of 0% obtained in this study is relatively close to the rates of ≤5% reported by some researchers, it is still a lower value compared to all previous studies in the literature to date. Further research is needed to determine whether this absence of PTF is a breed-specific characteristic of Turkish Kangal shepherd dogs or a finding unique to the present study.

It was previously reported that the average litter size of Turkish Kangal shepherd dogs ranges from 5.9 to 8.9 [23]. In our study, the mean litter size was found to be 9.2, slightly greater than the range stated in the literature.

Mila et al.[10] reported that the effects of variables such as breed size, sex, season of birth, colostrum IgG concentration, and litter size on passive transfer success in puppies were insignificant on a dog farm where different dog breeds were bred. In the present study, the effects of maternal age and the sex of the puppies on blood IgG levels at 2 days of age were similarly found to be insignificant for passive transfer success, while the effects of birth season and litter size were significant, in contrast to the findings of Mila et al.[10]. In this study, with a mean litter size of 9.2, the reason for the negative effect of increasing litter size on passive transfer success may be that the colostrum of dams with larger litters may not be sufficient for all of the offspring, while the colostrum of dams with 5 or fewer offspring is more likely to be sufficient and higher passive transfer success will likely be achieved. In addition, dams with 5 or fewer puppies have more opportunities to care for their offspring on an individual basis, whereas dams with larger numbers of puppies may be exhausted due to the prolonged birth process and more extensive feeding, leaving them unable to care for each puppy individually and negatively affecting the success of passive transfer. Considering the effect of the season of birth on passive transfer success, it was observed that the rate of passive transfer success was higher in winter compared to summer in the present study. This may have been due to the fact that the dams were more affected by heat stress in summer and processes requiring close physical contact such as feeding were kept to shorter durations as the dams tried to prevent their body temperatures from rising even

more, indirectly affecting the success of passive transfer. While the mean IgG levels in spring and fall were found to be quite close to the general mean value obtained in this study, they were highest in winter, when the dams were in constant contact with the newborn puppies to maintain their body temperatures. The dam's effort to remain in constant contact with the puppies in winter to protect them from hypothermia would give the puppies more opportunities to suckle during the course of that contact. However, this hypothesis can only be confirmed by future studies that compare ambient temperatures and suckling times of puppies in the first 48 h of life.

Mila et al.[10] evaluated the factors affecting neonatal mortality and found that the effects of the sex of the puppies, breed, colostrum IgG concentration, and litter size were insignificant while blood IgG levels at 2 days of age were significant (P=0.018). Tønnessen et al.[16] found the effect of maternal age on puppy mortality to be statistically significant. In the present study, individual follow-up was not possible because the puppies were not marked. In our study, the mortality rate of the offspring of 15 dams was obtained by calculating the ratio of the puppies surviving until weaning (2 months of age) to the total number of offspring included in the study. No correlation was found between passive transfer success and survival until weaning. However, Mila et al.[10] found a correlation between passive transfer success and mortality when they followed puppies individually and evaluated mortality within 21 days, in contrast to our study. Furthermore, our study was conducted under field conditions in an environment in which biosecurity measures were very limited, unlike the controlled study of Mila et al.[10], in which biosecurity measures were applied on a professional farm. This may explain the discrepancies between the results of these studies. Under field conditions, puppies are exposed to many factors that may increase the mortality rate, particularly including infective agents [16]. In the present study, since the 15 dams were divided into 4 age groups and there was a small number of dams in each group, the effect of maternal age on puppy mortality was not statistically evaluated. Tønnessen et al.[16] reported that maternal age had a significant effect on puppy mortality in their comprehensive study of 58.439 puppies born to 10.810 dams. The Turkish Kangal shepherd dogs were quite tolerant of the manipulations performed on their offspring during the present study and it is anticipated that the individual marking of puppies will not be a problem in future studies. In this way, it will be possible to follow the health parameters of individual puppies and compare them in terms of all variables, allowing more detailed and reliable results to be obtained.

Mila et al.[10] reported that 34 (17.43%) of 195 puppies born on a farm with dogs of different breeds died in the first 48 h after birth. The differences between the puppy mortality rates in this study and the rates of previous studies may be due to many different factors including farm and breeding conditions, litter size, and susceptibility to diseases according to the size of the breed [16].

The survival rate of Kangal puppies in our study was 34.78% at the end of 2 months, while Tepeli and Çetin [28] reported it as 87.05% and Kırmızı [24] reported it as 41% for the spring and summer months and 39.1% for autumn and winter. Oğrak [29] reported that this rate varied between 62.5% and 94.37% for puppies born in different months. These differences in the findings of the survival of Kangal puppies until weaning may be affected by variables including epidemics at different times and in different regions, care and feeding conditions, and the implementation of biosecurity measures. Although some of the studies discussed here were conducted on breeding farm, our study was conducted under field conditions with dogs fed by non-professional breeders where biosecurity measures were not applied, and so the higher mortality rates were an expected finding.

In conclusion the present study has offered the first evaluation of passive transfer success in Turkish Kangal shepherd dogs. It was found that the blood IgG levels of all surviving puppies were above the PTF threshold value, but further studies are needed to determine whether this is a result specific to the region or whether the passive transfer process is more successful for Kangal dogs than other breeds. It was also observed that passive transfer success was affected by season of birth and litter size, while maternal age and the sex of the puppies had no statistically significant effects. As another remarkable finding, although the passive transfer success rate was well above the average values, the puppy mortality rate was higher than the ranges reported in the literature data, revealing that dog owners who breed Turkish Kangal shepherd dogs, and particularly those around Kırıkkale, do not manage the processes of the neonatal period well and their biosecurity and management practices are inadequate. For this reason, it is necessary to raise awareness among the breeders in this region about neonatal protocols, the neonatal period, biosecurity, and preventive medicine practices that should be applied for puppies.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author.

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Ethical Statement

Kırıkkale University Animal Experiments Local Ethics Committee

approval was obtained before the study (Approval No. 2020(4)/25).

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Author Contributions

All authors contributed to the design of the study and the writing of the manuscript. EK, NÖ, SYD, and YŞ carried out blood sampling and health monitoring of the puppies. ÖD performed the laboratory analyses. NÖ performed the statistical analyses. All authors read and approved the final manuscript.

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Research Article

Comparative Analgesic Efficacy for Intraperitoneal Administration of Bupivacaine, Ropivacaine, and Ropivacaine-Tizanidine Combination During Ovariohysterectomy of Cats Suffering from Pyometritis

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Abstract

A prospective clinical trial was conducted on 30 cats suffering from pyometritis to investigate the multimodal analgesic efficacy of ropivacaine-tizanidine (RT) combination through intraperitoneal (IP) administration during ovariohysterectomy. Cats were randomly assigned into three groups whereby group RT received 0.5% ropivacaine (1 mg/kg) and tizanidine (10 µg/kg) (IP), group B received 0.5% bupivacaine (2 mg/kg) (IP), and group R received only 0.5% ropivacaine (1 mg/kg) (IP). It was observed that duration for anesthetic recovery (P<0.0001) and extubation time (P=0.0492) differed significantly for group RT. Body temperature and Pulse remained significantly higher in RT group at 1-, and 2-h postoperative intervals, specifically. Interactive Visual Analogue Scale (IVAS) and UNESP-Botucatu Multidimensional Composite Pain Scale (MCPS) scores were significantly lower for group RT at 6-, 8-, and 12-h time periods. Whereas, Mechanical nociceptive threshold (MNT) measurements differed significantly across 1 (P=0.0037), 4 (P=0.0013), 8 (P=0.0024) and 12-h (P=0.0258) time periods. The need for rescue analgesia and Serum cortisol concentrations, consistently remained significantly lower in group RT (P<0.0001). However, values for ALT, AST, ALP, BUN, and creatinine showed no significant differences among groups at 1-, 8-, and 24-h postoperative periods. The study concluded that the ropivacaine-tizanidine combination effectively reduced pain scores, delayed the need for rescue analgesia, and avoided adverse effects.

Keywords: Bupivacaine, Intraperitoneal instillation, Multimodal analgesia, Ropivacaine, Tizanidine, UNESP-Botucatu multidimensional composite pain scale

Introduction

Recent studies highlight the effectiveness of various multimodal analgesic strategies for mitigating postoperative pain and swelling in cats [1]. Among these approaches, intraperitoneal (IP) instillation of local anesthetic agents, emerges as a method capable of effectively managing postoperative pain over an extended duration, reducing the necessity for subsequent administration of anti-inflammatory drugs following major surgical procedures [2,3]. Previous research has established the efficacy of both bupivacaine and ropivacaine in providing prolonged postoperative pain relief with a minimal risk of systemic toxicity [2,4]. Additionally, investigations have compared the cardiotoxic and neurotoxic effects associated with intraperitoneal instillation of these agents as well [5,6]. A preliminary study suggests, that a multimodal approach involving intraperitoneal infusion of local anesthetic agents, could potentially prolong the effects of these drugs [2]. Achieving this would necessitate the concurrent use of α2-adrenergic agonists with these local anesthetics to not only block sensory receptors but also interrupt sensory reflexes

at the dorsal horn of the spinal column [1]. Tizanidine has been proposed as a suitable drug for directly inhibiting the cation current at the reflex arc level, significantly enhancing the duration and quality of analgesia [7]. The additional effect of tizanidine is speculated to induce localized vasoconstriction, thereby delaying the absorption of local anesthetics into the bloodstream [8]. The longer these agents remain in a circumscribed area, the more prolonged their effective duration of action would be [7]. Despite serious risks associated with systemic tizanidine administration, combining it with other medications or therapies at lower doses could enhance pain control for cats recovering from surgery or managing chronic conditions [9]. Unlike rats, pharmacodynamics of intraperitoneal tizanidine administration in cats has not been extensively researched. However, its efficacy in multimodal analgesic approaches when administered in lower doses has been suggested previously [10].

Recently it has been reported that intraperitoneal instillation of ropivacaine is not as efficacious as that of bupivacaine [11]. However, bupivacaine has been linked to greater instances of systemic toxicity, seizures, cardiac arrhythmias, and respiratory depression when administered intraperitoneally compared to ropivacaine [11,12]. Studies reveal that racemic compounds, such as Bupivacaine, exhibit higher absorptivity but may entail more adverse effects on canine or feline patients compared to the S (-) isomer, ropivacaine [2]. This discrepancy is attributed to the relatively lower lipophilicity of ropivacaine, resulting in milder effects on cardiac function [10]. However, this same characteristic makes ropivacaine less effective in blocking nerve impulses compared to bupivacaine [1]. Consequently, an intervention involving lower doses of tizanidine, in conjunction with ropivacaine could precisely enhance peri-operative analgesia in severely pyometric animals. This is particularly crucial given the significant abdominal distension caused by engorged uterine horns, leading to substantial regional discomfort post-excision [13]. Tizanidine was selected for its unique mechanism of action, perceived systemic safety, potential effectiveness, and its role in a multimodal pain management strategy, all with a primary focus on the well-being of feline patients. This study aimed to assess and compare the postoperative analgesic effects and potential adverse events resulting from the intraperitoneal instillation of bupivacaine, ropivacaine, and a combination of ropivacaine-tizanidine in pyometric cats undergoing ovariohysterectomy. The hypothesis posited by the authors was that the inclusion of tizanidine alongside ropivacaine would significantly enhance postoperative pain relief and prolong the time before rescue analgesia was required, all without adversely affecting liver and kidney functionality.

Material and Methods

Ethical Consideration

Only clinical patients requiring ovariohysterectomy for clinical imperatives were inducted into the study following provision of an informed consent from their respective caretakers. The design of this study followed guidelines outlined by the "Guide for the Care and Use of Laboratory Animals in Research and Teaching". Experiment was conducted under the auspices prescribed by the Ethical Review Committee of the Office of Research, Innovation & Commercialization (ORIC), Cholistan University of Veterinary and Animal Sciences, Bahawalpur-Pakistan (Approval no: ORIC 224) and was legally compliant with Punjab Wildlife Protection, Preservation, Conservation and Management Act (1974).

Selection of Animals

This research, conducted with informed consent from the owners, involved 30 domestic cats of various breeds diagnosed with pyometritis and deemed suitable for ovariohysterectomy for their survival. Preoperative abdominal ultrasonography was performed to exclude pregnancy and confirm pyometritis. The selected cats had an average age and body weight of 16.83±2.92 months and 2.59±0.13 kilograms, respectively. Cats were fasted overnight, and any severely emaciated or unwell patients were excluded. Pregnant, lactating, and fractious animals were also excluded from the study. All cats enrolled in the study were admitted at least 6 h before surgery.

Surgical Groups

Thirty cats inducted into this study were randomly allocated into three experimental groups. Cats in the first group were instilled with a combination of 0.5% ropivacaine (Ropicain; Howards®) at 1 mg/kg and tizanidine (Movax; Sami Pharmaceuticals, Pakistan) at 10 µg/kg (group RT, n = 10) intraperitoneally. A second group of individuals received 0.5% bupivacaine (Bupicain; Lahore Chemical & Pharmaceutical, Pakistan®) at 2 mg/kg (group B, n = 10) intraperitoneally, while a third group was administered with 0.5% ropivacaine (Ropicain; Howards®) at 1 mg/kg (group R, n = 10) during ovariohysterectomy of pyometric animals. Ten 2 mg tizanidine tablets were dissolved in 100 mL of sterile saline, resulting in a 0.02% solution. This solution was administered intraperitoneally at a dose rate of 10 µg/kg, with each drop of the solution roughly measuring 0.05 mL. The number of drops was calculated based on each patient's individual body weight and then added to the mixture to be instilled. For consistency, all solutions containing 0.5% bupivacaine, 0.5% ropivacaine, or a combination of ropivacaine and tizanidine were uniformly reconstituted with normal saline, resulting in a final volume of 1 mL. These preparations were

administered intraperitoneally, succinctly following the placement of ligatures around ovarian pedicle and uterine stump [14]. Considering the ethical implications, to avoid any unfortunate intra-operative pain, meloxicam at 0.03 mg/kg was administered to all cats before anesthetic induction [2].

Anesthetic and Surgical Procedures

The same anesthetist performed all the procedures who was blinded to the animal grouping. A 24-gauge intravenous catheter was aseptically placed in cephalic vein of all the presented patients. An adequate dose of propofol at 6 mg/kg (Diprivan; ICI, Pakistan®) was administered intravenously (IV) to induce sedation and allow for the placement of an endotracheal tube [6]. The endotracheal tube was attached to a non-rebreathing Bain system and isoflurane (Forane, Baxter Healthcare Corporation®) was maintained at a 1.58% Minimum alveolar concentration (MAC) value. Lactated Ringer's solution (Unisol-RL, UNISA Pharmaceutics, Pakistan®) was administered intravenously at 10 mL/kg/h until extubating. Electrocardiography, heart rate, respiratory rate, pulse oximetry, and plethysmography were repeatedly observed during anesthetic duration by employing a multiparametric monitor. A sphygmomanometer with cuff size number 1 was placed at the animal's antebrachium to monitor systolic arterial pressure (SAP). MAC value was adjusted based on the values of physiological parameters (body temperature, pulse rate, breathing rate, systolic arterial blood pressure) and anesthetic reflexes (medio-ventral rotation of the eyeball, loss of jaw tone and palpebral reflexes). Ovariohysterectomy was performed employing a typical technique whereby surgical approach was made through a midline laparotomy incision in dorsally recumbent cats. Same surgeon performed all procedures to alleviate any discrepancies based on the differences in skill and experience. The anesthetic duration was determined by calculating the time elapsed since the induction by propofol till the discontinuation of isoflurane. Whereas the time it took for the surgeon to give primary incision up until the placement of final sutures indicated the duration of surgery. As animals started to recover and a swallowing reflex was observed, they were extubated and time elapsed since termination of isoflurane was noted as well. While overall recovery time was estimated when patients voluntarily resumed their sternal positions.

Scoring for Pain

Trained observers, blinded to the treatment groups and experienced in pain assessment, were designated to assign pain scores. The postoperative impact of drug combinations on animal consciousness and responsiveness was evaluated using an Interactive Visual Analogue Scale (IVAS). In this scale, an elevated pain sensation was indicated by a higher numerical figure on the 0-100 equidistant lines. Additionally, a more comprehensive UNESP-Botucatu Multidimensional Composite Pain Scale (MCPS) ranging from 0 (no pain) to 30 (maximum pain) was employed to subjectively assess the effectiveness of analgesic regimens. Baseline values were established 6 h before surgery, and subsequent readings were recorded at 1, 2, 4, 6, 8, and 12 h post-extubation for both scales. Animals were coerced to move about, inside the cage, once they had recovered from anesthesia. The peri incisional area was palpated and mechanical nociceptive thresholds (MNT) was assessed using homemade von Frey filaments as described by de Sousa et al.[15]. Probing was performed intermittently (6 h pre-op, 1 h, 4 h, 8 h, and 12 h) using Nylon filaments around peri-incisional region, exerting incremental levels of force (0.5, 2.0, 20.0, 39.0, 78.0, 98.0 mN).

Rescue Analgesia and Other Adverse events

Rescue analgesia was accomplished using buprenorphine at 0.01 mg/kg when MCPS was ≥6, whereas other adverse events namely vomiting, diarrhea, seizures, and cardiovascular incidences were recorded as well. Individuals requiring rescue analgesia or that experienced any complications were recorded and subsequently removed from the study as no further observations were made for them.

Laboratory Testing for Blood Glucose and Serum Cortisol Concentrations

Blood glucose and Serum cortisol concentrations could prove useful as biochemical indicators of inflammation and pain. 6 h before surgery and then at 1, 8, and 24 h post-operatively, blood samples were collected for the evaluation of blood glucose and serum cortisol concentrations. Blood glucose concentrations were estimated in mg/dL using a glucometer while collected serum was shipped to a laboratory for serum cortisol analysis using solid phase radioimmunoassay.

Biochemical Testing for Liver and Kidney Function

Blood samples were obtained at various time points i.e., 6 h before surgery and at 1, 8, and 24 h following the procedure. These samples were allowed to coagulate and were then subjected to centrifugation at 3000 rpm for 15 min to separate the serum. The resulting serum was meticulously preserved at -20°C, awaiting subsequent biochemical analysis. Feline liver function was assessed by estimating Alanine aminotransferase (ALT (U/L)), Aspartate transaminase (AST (U/L)), and Alkaline phosphatase (ALP (U/L)) [16]. Whereas, Blood urea nitrogen (BUN (mg/dL)) and Creatinine (mg/dl) were investigated for ascertaining kidney function (Vet Scan VS2 analyzer, ABAXIS®, USA).

Statistical Analysis

Values pertaining to parametric variables such as age, weight, physiological parameters, duration of anesthesia, duration of surgery, extubation time, recovery times, serum cortisol and plasma glucose concentrations were presented as mean ± standard deviation and compared among groups using one-way analysis of variance followed by a Bonferroni test. Whereas nonparametric variables namely UNESP-Botucatu multidimensional composite pain scale scores and mechanical nociceptive threshold values were analyzed using a Kruskal-Wallis's test. Fisher's exact probability test was used to compare number of cats requiring rescue analgesia. Biochemical parameters namely AST, ALT, ALP, BUN and creatinine were also compared for experimental groups using a one-way analysis of variance followed by a pairwise comparison by Dunn's test. All analyses were performed using GraphPad Prism (version 8.4.3) and statistical significance was indicated when $P<0.05$.

Results

Demographic parameters namely, age (range: 11-23 months) (P=0.8270) and body weight (range: 2.4-2.8 kg) (P=0.1470) estimated before the initiation of clinical trial, were statistically non-significant between RT, B, and R groups. This was done to verify the randomness of grouping process and ensure elimination of experimental bias during allocation of individuals into different groups.

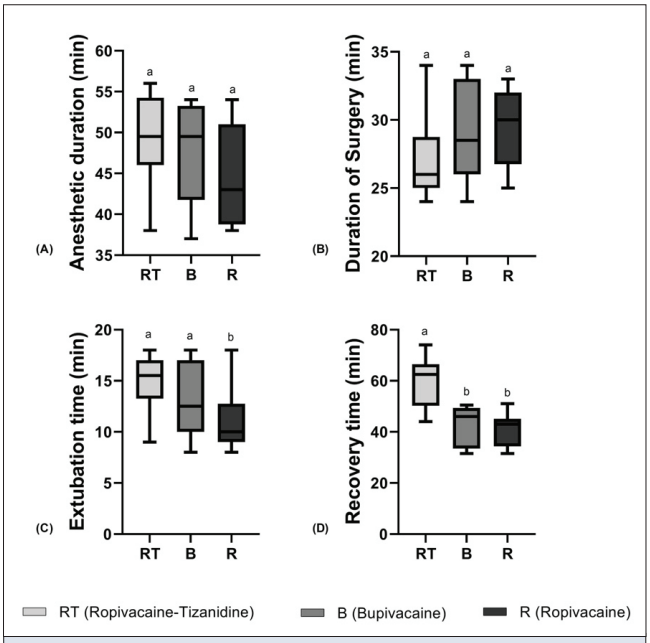

Fig 1. Values for different intraoperative variables represented as Box and Whisker plots in Groups RT, B and R: (A) Average duration of anesthesia (min); (B) Duration of Surgery (min); (C) Extubation time (min); and Recovery time (min) amongst experimental groups (Groups RT, B and R). Whereby significant differences ($P<0.05$) among groups are indicated by different superscripts [(a,b,c)]

Operative durations including surgical (P=0.2547) and anesthetic (P=0.2408) durations were also ensured to be non-significant as a comparable technique was employed by same personnel for all patients. Whereas, duration for anesthetic recovery was significantly different amongst groups when a multiple comparison post-hoc test was performed between ropivacaine-tizanidine group, bupivacaine group (P=0.0001), and ropivacaine group (P=0.2408). Moreover, extubation time was significantly different between group RT and R (P=0.0492) as well *(Fig. 1)*.

All parameters indicating physiological normalcy i.e., body temperature (°F), pulse (beats/min), respiration (breaths/min) and systolic arterial blood pressure (mmHg) were determined 6 h prior to anesthesia to establish base line values for all individuals. No statistical difference was reported amongst any of the groups. Observations were repeated at 1-, 2-, 4-, 6-, 8-, and 12-h intervals postoperatively as well. At 1h postoperatively body temperature was observed to be significantly higher in RT group when compared with B (P=0.0010) and R (P=0.0113). A similar pattern was reported at 2- and 4-h intervals as well, whereby values in RT group remained higher *(Fig. 2)*. While values between groups were non-significant at 6-, 8- and 12-h marks. Pulse rate was observably increased amongst all the study groups relative to their baseline values, but values amongst individuals of RT group were significantly higher at 1 h and 2 h post-operatively whereas with the progression of time this trend changed and by 6th h B group experienced observably higher pulse rates. Respiration rates remained non-significant across all groups throughout the experimental design. While systolic arterial pressure was significantly

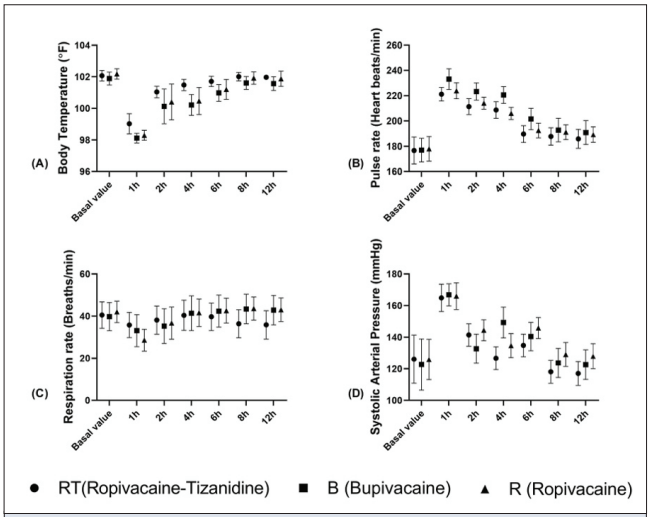

Fig 2. Interleaved symbols graph illustrating Pre- and Post-operative values of: (A) Body temperature (°F); (B) Pulse rate (Heart beats/min); (C) Respiration rate (Breaths/min); and (D) Systolic arterial pressure (mmHg) in surgical patients (Groups RT, B, and R) at Basal value (preoperative assessment 2 h before surgery), 1 h, 2 h, 4 h, 6 h, and 8 h postoperatively. Whereby values are represented as Mean and error bars indicate ±SD

Table 1. *Pain scores for Interactive visual analogue scale (IVAS), Modified Composite Analogue Scale (MCPS) and Mechanical Nociception Test (MNT) represented as median (Range)*

Pain Scales	Groups	Time Interval (hours)						
		Preoperative (6h)	Postoperative					
		Baseline (BL)	1h	2h	4h	6h	8h	12h
IVAS (0-100)	RT (n=10)	0 (0-0) [a]	3.5 (0-15) [a]	5.5 (0-16) [a]	7.5 (0-17) [c]	9.5 (0-22) [c]	14.5 (0-37) [c]	16.5 (12-30) [c]
	B (n=10)	0 (0-0) [a]	7.5 (0-25) [a]	13 (0-26) [a]	17.5 (0-36) [b]	20 (2-30) [b]	28 (3-38) [b]	33 (11-40) [b]
	R (n=10)	0 (0-0) [a]	18.5 (0-30) [a]	19 (0-30) [a]	28.5 (0-38) [a]	29.5 (19-37) [a]	37 (26-40) [a]	44 (31-49) [a]
MCPS (0-30)	RT (n=10)	0 (0-0) [a]	1 (0-2)	1 (0-2)	1 (0-3)	1 (0-3)	3 (1-8)	3 (9-1)
	B (n=10)	0 (0-0) [a]	1.5 (0-4)	2 (0-4)	2 (0-8)	3 (0-5)	7 (2-13)	8 (3-14)
	R (n=10)	0 (0-0) [a]	2 (0-4)	2 (0-5)	3 (0-7)	3 (0-6)	12 (2-16)	15 (14-21)
MNT (mN)	RT (n=10)	98 (98-98) [a]	98 (98-98)	---	98 (78-98)	----	88 (39-98)	88 (39-98)
	B (n=10)	98 (98-98) [a]	88 (78-98)	---	78 (20-98)	---	39 (20-78)	39 (20-78)
	R (n=10)	98 (98-98) [a]	78 (20-98)	---	58.5 (2-98)	---	29.5 (2-39)	20 (2-39)

Group RT (0.5% Ropivacaine at 1 mg/kg and Tizanidine at 10 µg/kg); Group B (0.5% Bupivacaine at 2 mg/kg); Group R (0.5% Ropivacaine at 1 mg/kg). Statistical significance (P<0.05) amongst prospective groups is identified by different superscripts [a,b,c] in a column

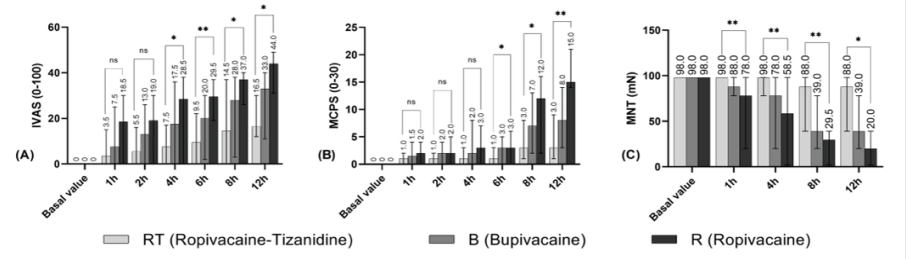

Fig 3. Interleaved bar graphs illustrating mean pain scores: (A) Interactive visual analogue scale (IVAS); (B) Modified Composite Analogue Scale (MCPS); and (C) Mechanical Nociception Test (MNT) represented as median (Range), observed 6 h prior to surgery (baseline) and subsequently at 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h periods postoperatively in 30 cats instilled intraperitoneally with 0.5% Ropivacaine at 1 mg/kg and Tizanidine at 10 µg/kg (group RT, n=10); 0.5% Bupivacaine at 2mg/kg (group B, n=10); or 0.5% Ropivacaine at 1 mg/kg (group R, n=10). Whereby significant differences (P<0.05) among groups are indicated by different superscripts [a,b,c]

higher in group RT as opposed to group B (P<0.0001) at 4-h mark, but continued to remain different from only group R, at subsequent time intervals *(Fig. 2)*.

When compared with baseline IVAS scores were observably higher in subsequent postoperative periods. At 4-, 6-, 8-, and 12-h time intervals the values amongst groups were statistically different from each other whereby P=0.0279, P=0.0014, P=0.0134 and P=0.0138 respectively *(Table 1)*.

A similar trend was observable in the case of MCPS values, whereby significant difference was estimated at 6 (P=0.0193), 8 (P=0.0457) and 12 h (P=0.0011) *(Fig. 3)*. However, the MNT measurements differed significantly across 1 (P=0.0037), 4 (P=0.0013), 8 (P=0.0024) and 12-h (P=0.0258) time periods *(Fig. 3)*.

Number of cats requiring rescue analgesia were significantly lower for group RT as opposed to other treatment groups (P<0.0001). In case of group B, rescue analgesia was administered to 1, 5 and 4 cats at 4-h, 8-h and 12-h respectively. While 2 cats at 4-h, 1 cat at 6-h, 4 cats at 8-h, 3 cats at 12-h mark had to be rescued in case of group R. No serious complications or other adverse events were observed during the period of this study. Elevated serum cortisol levels were reported for all groups post-operatively, however, greatest cortisol values were observed in patients of group R while the lowest were seen for group RT (P<0.0001) at 1 h mark postoperatively *(Fig. 4)*. The numerical differences continued to decrease by 8 h and 24 h periods. Blood glucose concentrations were statistically significant across all time periods between different groups (P<0.0001) *(Fig. 4)*.

Biochemical parameters namely, alanine aminotransferase (ALT (IU/L)), aspartate transaminase (AST (IU/L)), alkaline phosphatase (ALP (IU/L)), blood urea nitrogen (BUN (mg/dL)) and creatinine (mg/dL) were compared for experimental groups using a one-way analysis of variance *(Fig. 5)*.

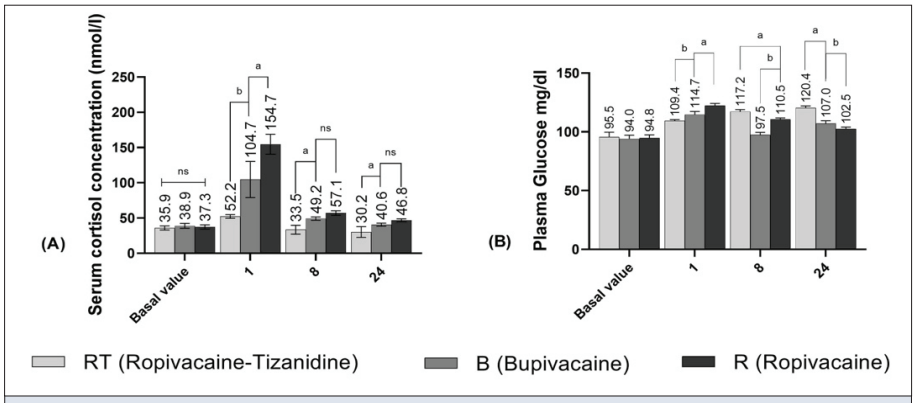

Fig 4. Interleaved bars illustrating: (A) Serum cortisol concentrations (nmol/L); and (B) Plasma Glucose concentration (mg/dL) as (Mean ± SD) values, observed 6 h prior to surgery (baseline) and subsequently at 1 h, 8 h, and 24 h periods postoperatively in 30 cats instilled intraperitoneally with 0.5% Ropivacaine at 1 mg/kg and Tizanidine at 10 µg/kg (group RT, n=10); 0.5% Bupivacaine at 2 mg/kg (group B, n = 10); or 0.5% Ropivacaine at 1 mg/kg (group R, n=10). Whereby significant differences (P<0.05) among groups are indicated by different superscripts (a,b,c)

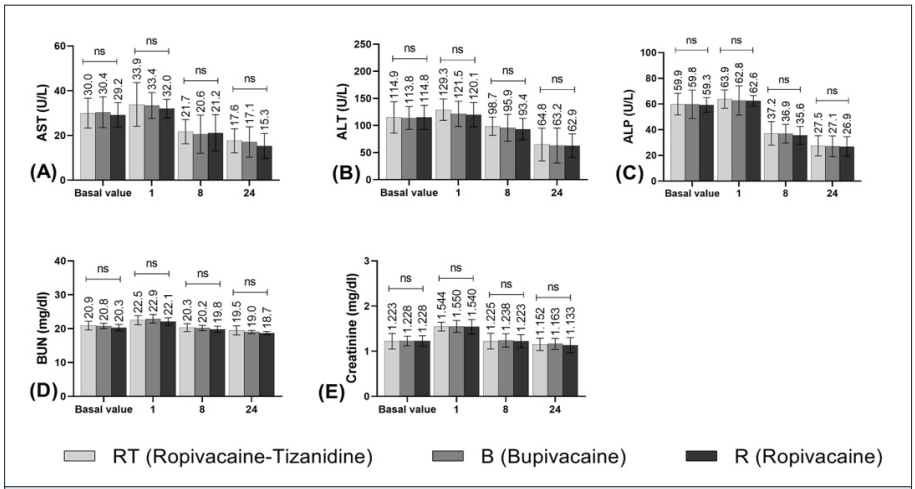

Fig 5. Interleaved bars illustrating: (A) Aspartate transaminase (AST (U/L)); (B) Alanine aminotransferase (ALT (U/L)); (C) Alkaline phosphatase (ALP (U/L)); (D) Blood urea nitrogen (BUN (mg/dL)) and (E) Creatinine (mg/dL) as (Mean ± SD) values, observed 6 h prior to surgery (baseline) and subsequently at 1 h, 8 h, and 24 h periods postoperatively in 30 cats instilled intraperitoneally with 0.5% Ropivacaine at 1 mg/kg and Tizanidine at 10 µg/kg (group RT, n=10); 0.5% Bupivacaine at 2 mg/kg (group B, n=10); or 0.5% Ropivacaine at 1 mg/kg (group R, n=10). Whereby significant differences (P<0.05) among groups are indicated by different superscripts (a,b,c)

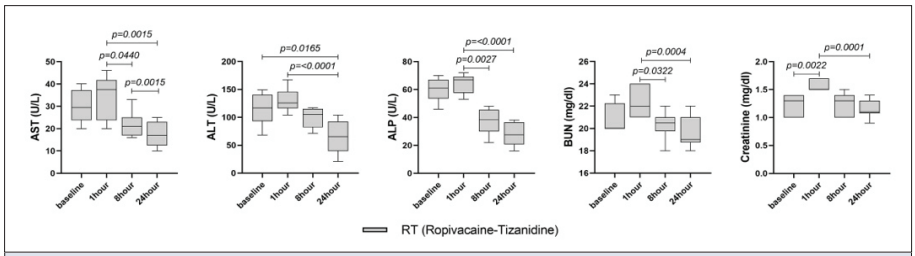

Fig 6. Box and whisker plot illustrating pair wise analysis of group RT (Ropivacaine-Tizanidine) using Dunn's multiple comparisons test for biochemical parameters namely Aspartate transaminase (AST (U/L), Alanine aminotransferase (ALT (U/L), Alkaline phosphatase (ALP (U/L), Blood urea nitrogen (BUN (mg/dL)) and Creatinine (mg/dL) observed 6 h prior to surgery (baseline) and subsequently at 1 h, 8 h, and 24 h periods postoperatively in 30 cats

A post-hoc Bonferroni test was also performed for multiple comparisons amongst groups. None of the variables were statistically different for Group RT, B and R, at baseline, 1, 8 or 24 h of the experimental period *(Table 2)*.

Pairwise analyses within groups were conducted using Dunn's multiple comparison test. In the RT, B, and R groups, all parameters, including ALT, AST, ALP, BUN, and creatinine, exhibited statistically significant differences

Table 2. *Biochemical Parameters for Liver and Kidney function represented as mean ± SD*

Biochemical Parameters for Liver and Kidney Function	Groups	Time Interval (hours)			
		Preoperative (6h)	Postoperative		
		Baseline (BL)	1h	8h	24h
Aspartate transaminase (AST, U/L)	RT (n=10)	30±6.7 ^{aA}	33.9±9.8^{aA}	21.7±5.4 ^{aB}	17.6±5.4 ^{aB}
	B (n=10))	30.4±6.88 ^{aA}	33.4±5.8 ^{aA}	20.6±8.5 ^{aB}	17.1±6.8 ^{aB}
	R (n=10)	29.2±5.45 ^{aA}	32±4.19 ^{aA}	21.2±8.1 ^{aB}	15.3±5.6 ^{aB}
Alanine aminotransferase (ALT, U/L)	RT (n=10)	114.9±29 ^{aA}	129.3±19 ^{aA}	98.7±17 ^{aA}	64.8±30 ^{aB}
	B (n=10)	113.8±21 ^{aA}	121.5±23 ^{aA}	95.9±25 ^{aA}	63.2±32 ^{aB}
	R (n=10)	114.8±22 ^{aA}	120.1±22 ^{aA}	93.4±20 ^{aA}	62.9±22 ^{aB}
Alkaline phosphatase (ALP, U/L)	RT (n=10)	59.9±8.4 ^{aA}	63.9±7.1 ^{aA}	37.2±9.1 ^{aB}	27.5±7.9 ^{aB}
	B (n=10)	59.8±11.1 ^{aA}	62.8±11.4 ^{aA}	36.9±7.3 ^{aB}	27.1±8.1 ^{aB}
	R (n=10)	59.3±5.64 ^{aA}	62.6±4 ^{aA}	35.6±7 ^{aB}	26.9±7.6 ^{aB}
Blood urea nitrogen (BUN, mg/dL)	RT (n=10)	20.9±1.3 ^{aA}	22.5±1.4 ^{aA}	20.3±1.2 ^{aA}	19.5±1.4 ^{aB}
	B (n=10)	20.8±0.79 ^{aA}	22.9±1.3 ^{aA}	20.2±0.8 ^{aB}	19±0.5 ^{aB}
	R (n=10)	20.3±0.95 ^{aA}	22.1±1.1 ^{aA}	19.8±0.9 ^{aB}	18.7±0.5 ^{aB}
Creatinine (mg/dL)	RT (n=10)	1.22±0.17 ^{aA}	1.54±0.1 ^{aB}	1.23±0.2 ^{aC}	1.15±0.1 ^{aC}
	B (n=10)	1.23±0.11 ^{aA}	1.55±0.3 ^{aB}	1.24±0.2 ^{aC}	1.16±0.1 ^{aC}
	R (n=10)	1.23±0.12 ^{aA}	1.54±0.16 ^{aB}	1.22±0.14 ^{aC}	1.13±0.17 ^{aC}

Group RT (0.5% Ropivacaine at 1 mg/kg and Tizanidine at 10 µg/kg); Group B (0.5% Bupivacaine at 2 mg/kg); Group R (0.5% Ropivacaine at 1 mg/kg). Statistical significance (P<0.05) amongst prospective groups is identified by different superscripts $^{(a,b,c)}$ in a column. Whereas, Statistical significance (P<0.05) within groups at different time periods resulting from Dunn's multiple comparison tests, are denoted by different superscripts $^{(A,B\,C)}$ in a row

between baseline and 24 h postoperatively, with baseline values being notably higher *(Table 2)*. In the RT group, AST values showed significant differences between 1 and 8 h (P = 0.0440) and between 8 and 24 h (P=0.0015). Meanwhile, ALT values displayed significant differences at both baseline (P=0.0165) and 1 h (P<0.0001) when compared with the 24-h samples. ALP values followed a similar pattern to AST, with statistical differences observed between 1 and 8 h (P=0.0027) and 1 and 24 h (P<0.0001) *(Fig. 6)*. Both BUN (P=0.0004) and creatinine (P=0.0001) levels were statistically different between 1 and 24 h postoperatively.

Discussion

In our study, combining tizanidine with ropivacaine intraperitoneally during ovariohysterectomy of pyometric cats, significantly improved analgesic efficacy. This approach aimed to prolong the time before rescue analgesia and minimize adverse events associated with instilling bupivacaine or ropivacaine alone. The results of this study corroborated prior findings whereby addition of an alpha 2-agonist to a local instillation protocol was employed to produce a pronounced analgesic event [2]. Previously, when two intraperitoneal (IP) regimens of 0.25% bupivacaine-dexmedetomidine and bupivacaine-epinephrine were compared, researchers observed similar postoperative pain scores. However, they noted a general reduction in the overall frequency of rescue analgesia events for the bupivacaine-dexmedetomidine group, indicating improved efficacy following the addition of an alpha-2 agonist [17]. Similarly, in current settings, authors have observed a positive response in terms of both the postoperative pain scores and rescue analgesic events. A contemporary finding has revealed lower efficacy of Ropivacaine as compared to Bupivacaine in dogs. Out of 22 dogs instilled intraperitoneally with 0.5% bupivacaine, only 6 required rescue analgesia. [11]. Similar outcomes were observed by authors in our study as well, where rescue analgesia was administered to 1, 5 and 4 cats at 4-h, 8-h and 12-h respectively in case of 0.5% bupivacaine while 2 cats at 4-h, 1 cat at 6-h, 4 cats at 8-h, 3 cats at 12-h mark had to be rescued in groups administered with 0.5% bupivacaine. Nevertheless, despite exhibiting identical outcomes, both studies were quite different in terms of experimental design, clinical settings and experimental species. Several other discrepancies observed in our study as opposed to contemporary findings could be rationalized by the inherent differences in experimental approaches [4,17].

Authors employed two different scoring systems to evaluate the post operative analgesic ability of ropivacaine

and tizanidine, namely IVAS and MCPS [2]. Prior investigations have indicated that bupivacaine proved to be a better alternative to ropivacaine when administered intraperitoneally [17]. However, the toxicity and other adverse reactions associated with bupivacaine have deemed it desirable to investigate alternatives. Ropivacaine could prove an alternative but its ability to affect psychomotor functionality is quite limited [12]. Thereby, leading researchers to conduct experiments using various concoctions made with alpha-2 agonists [17]. The assessment of pain in cats in this study focused on two domains, pain expression and psychomotor changes [12]. Due to instrumental limitations, specifically the lack of an electroencephalogram (EEG) for brain wave mapping, the ability to draw conclusions regarding psychomotor changes was restricted. Therefore, the current study relied heavily on physiological norms (temperature, pulse, and respiration) and pain scores for making inferences. The estimation of sedation, depth and degree of consciousness heavily relied on IVAS scores, while composite pain scores served as the primary indicators for evaluating postoperative pain [18]. Consequently, the assessors had to be adequately trained beforehand to properly gauge the degree of pain, considering the highly subjective nature of these scales [19]. In the present study, the relatively low pain scores observed across individuals in all experimental groups could be attributed to comparable study conditions and the expertise of an experienced surgeon who performed all the surgeries with minimal tissue trauma. However, it should be noted that the low pain scores for IVAS and MCPS within the first 4 h after surgery may have made it challenging for the assessors to differentiate among groups, as the overall scores were exceptionally low [1]. On the other hand, objective parameters such as nociception threshold scores (MNT) showed noticeable differences among groups. Overall, these findings highlight the importance of considering both subjective and objective measures when assessing pain in cats and emphasize the need for further investigation in this area [2].

Rescue analgesia for all individuals was managed using a single dose of buprenorphine at 0.01 mg/kg [17,20]. However, prior investigations have also employed short-acting opioids such meperidine for similar purposes [2]. To approximate the experimental design with everyday clinical situations and due to ethical concerns about inefficacy of our intraperitoneal instillations in controlling intraoperative pain, meloxicam was administered to all cats before surgery. Meloxicam has been commonly used for its analgesic properties in cats undergoing various surgical procedures, including ovariohysterectomy (spay) [21]. However, it is important to consider potential effects on clotting when using meloxicam as a premedication [1]. It has also been reported to exert a potent anti-hyperalgesic effect, leading to reduced behavioral responses following ovariohysterectomy [22]. Moreover, local anesthetics, namely bupivacaine and ropivacaine, have the potential to induce cardiac side effects, including rare instances of conduction disturbances, such as AV blocks [6]. Similar to previous studies, authors observed development of AV blocks in 2 of the cats receiving Ropivacaine IP instillation [10]. The incidence of these effects in cats is considered to be very low, however, it's important to note that individual responses to medications can vary, and certain factors such as the dose, concentration, and rate of administration may influence the risk of adverse effects [12]. Additionally, pre-existing cardiac conditions or concurrent administration of other medications may also increase the potential for cardiac complications. Therefore, in our current study both bradycardia and AV block were considered minor complications and did not require specific treatment.

Unlike these amide-type local anesthetics, tizanidine is an alpha-2 agonist notorious for inducing severe side effects in humans [23]. Tizanidine is a muscle relaxant primarily used in humans [7]. A small number of cats have reportedly exhibited hypersensitivity towards tizanidine and other similar drugs from same class [24]. Notably, tizanidine undergoes liver metabolism, making cats with pre-existing liver issues less suitable candidates for its use [24]. However, research has shown that hepatic toxicity is less common in cats than in humans [25]. Some cats may experience nausea, vomiting, diarrhea, and cardiac arrythmias when taking tizanidine, but these issues have been related to the use of all α2-adrenergic class agonists [26]. Most of these concerns were associated with prolonged usage and higher dose rates of at least 25-50 µg/kg [27]. Researchers have postulated that tizanidine could be considered in cats experiencing severe muscle spasms or spasticity due to neurological or musculoskeletal conditions. It could provide relief by reducing excessive muscle contractions [27]. Nevertheless, in the current study, the authors conducted a comprehensive risk-benefit analysis and hypothesized that employing a lower, one-time dose of tizanidine would not only effectively manage the spasticity [7] of abdominal musculature following the removal of an engorged uterus but also alleviate the neuralgia [24] associated with the transection of the ovarian pedicle. Hence, in order to confirm the safety of short-term intraperitoneal administration of tizanidine in cats, both preoperatively (6 h prior) and postoperatively (at 1, 8, and 24 h), serum biochemistry assessments were performed to examine the levels of AST, ALT, ALP, BUN, and creatinine. These biochemical parameters have been considered as effective biomarkers for assessment of hepatic and renal function [28]. In the current study, the authors observed noteworthy deviations in the mean baseline values of AST, ALT, and ALP from the

established reference values [16], while BUN and creatinine exhibited relatively minimal variation. Previous research has indicated that in cases of pyometra, bacteria present in the infected uterus may release harmful toxins into the bloodstream [29], potentially affecting liver function and giving rise to a condition referred to as toxic liver syndrome. Furthermore, substantial losses of electrolytes and fluids through discharge, along with reduced blood flow to the kidneys due to a shared vascular supply, could contribute to the development of acute kidney injury [30]. Authors observed that none of the variables were statistically different for Group RT, B and R, at baseline, 1, 8 or 24 h of the experimental period, indicating that there was no additional deleterious consequence of including tizanidine to local anesthetic concoctions. The subjects enrolled in the current study were confirmed to have pyometritis through clinical observations and ultrasonography. Consequently, it was anticipated that hepatic and renal function might be compromised, and this expectation was substantiated when baseline values were found to be notably higher than the reference range [16]. However, consistent with findings from prior investigations, following the successful completion of ovariohysterectomy, values of AST, ALT, ALP, BUN, and creatinine steadily decreased over time, eventually falling well within the safety parameters [29,30]. This observation underscored that neither the intraperitoneal administration of tizanidine in low doses nor that of amide local anesthetics had a significant impact on the liver and kidney functionality of the patients.

The authors faced a potential constraint regarding the dose rates of tizanidine, given the scarcity of in-depth research on this drug in cats [24]. Additionally, converting tizanidine from tablet form to a solution may have affected its physical and chemical properties, potentially impacting its onset of action, duration, and effectiveness in blocking sensory responses. However, it is important to note that no significant signs of systemic toxicity were observed at the dose and concentration of tizanidine utilized in this study. Tizanidine, being a monoisotopic drug has an absolute oral bioavailability of approximately 40% [31]. About 30% of the drug binds to plasma proteins, and a substantial portion undergoes hepatic metabolism primarily via CYP1A2 [32]. In capsule or tablet formulations, the active ingredient is presented as tizanidine hydrochloride (DS 103-282) [33]. These formulations contain minimal amounts of inactive ingredients like Anhydrous lactose and Microcrystalline cellulose, resulting in a relatively water-soluble form (0.133 mg/mL) [31]. The decision to reconstitute tizanidine tablets in sterile saline was in line with prior research, particularly in rodent models [34-37], where researchers demonstrated the feasibility and effectiveness of intraperitoneal delivery. The rationale for this method was grounded in the higher bioavailability associated with intraperitoneal administration, bypassing the first-pass metabolism in the liver [38]. Although existing literature predominantly discussed such practices in rodent studies, our findings emphasize the necessity for further exploration in feline medicine.

In conclusion, as part of a multimodal pain therapy for cats undergoing ovariohysterectomy, instilling ropivacaine-tizanidine onto the ovarian pedicles and uterine body after excision resulted in significantly pronounced postoperative analgesic effects without exhibiting any pronounced hepatic or renal adverse reactions. However, further investigations may be needed to identify the analgesic efficacy of tizanidine as analgesic treatment in cats.

Availability of Data and Materials

The authors declare that the experimental data supporting the present study findings have been made available to the corresponding author (A.H. Rabbani).

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Conflict of Interest

I hereby declare that there are no conflicts of interest associated with this research. The study is purely academic and aims to contribute to the existing knowledge in the field of veterinary medicine.

Author Contributions

Experimental Design was conceived by AHR, ON and KH. Data was collected by AHR, MS, and QU. Statistical analysis was conducted by ASA and MLS. Original draft was written by AHR and FW. Revision and final proof-reading of the manuscript were accomplished by ON, KH, and FW.

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Research Article

Adenylate Cyclase Affects the Virulence of Extraintestinal Pathogenic Escherichia coli Derived from Sheep Lungs

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Abstract

Escherichia coli is an important component of the normal bacterial community in humans and animals. However, in recent years, the pathogenicity of some virulent strains to the outside of the intestine has been confirmed in clinical medicine. The purpose of this study was to analyze the effect of *cyaA* gene knockout on the biological characteristics and pathogenicity of extraintestinal pathogenic *E. coli*. In this study, ExPEC strain (XJ10) was isolated from the lung tissue of sheep with respiratory tract infection, and the mutation strain and complementary strain of *cyaA* gene were constructed. Real-time PCR analysis showed that expression levels of most genes related to carbon source utilization and adhesion invasion were down-regulated in the *cyaA* mutant compared to the wild type; Quantification of the number of colonies showed that *cyaA* mutation results in the number of adhesion and invasive to TC-1 cells colonies decreased compared with wild type; Finally, as the *cyaA* gene mutated, the value of the LD_{50} increased as compared to wild type, based on the LD_{50} calculation. Taken together, mutations in the *cyaA* gene reduce growth rates, resulting in down-regulation of the transcription levels of genes associated with carbon source utilization and adhesion invasion, and a marked reduction in virulence.

Keywords: Adhesion and invasion, *cyaA*, Median lethal dose, XJ10

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Introduction

Escherichia coli can be divided into entero-pathogenic *E. coli* and extra-intestinal pathogenic *E. coli* according to their pathogenic characteristics. Through adaptive changes, Extraintestinal Pathogenic *E. coli* (ExPEC) have developed the ability to colonize extra-intestinal regions, causing pathologies in multiple organs and leading to diseases in humans and animals, including meningitis, septicemia, urinary tract infections, pneumonia, avian perihepatitis, and peritonitis. These infections are a serious human public health threat and thus have attracted the attention of researchers in China and abroad [1-3]. ExPEC can cause widespread infections in humans and animals, which are closely related to the presence of numerous virulence factors such as adhesins, toxins, protective factors, iron uptake systems, and pathogenicity islands. Each of these factors plays a different role during the different stages of infection [4-7].

Adenylate cyclase (AC), encoded by the *cyaA* gene, can enter a variety of eukaryotic cell and is mainly distributed on the plasma membrane, nuclear membrane, and endoplasmic reticulum membrane. When a cell receives a foreign signal molecule, the signal molecule binds to the corresponding membrane G protein-coupled receptors

(GPCRs), thereby activating AC. Upon activation of the *cyaA* gene, ATP is catalyzed to form cyclic adenosine monophosphate (cAMP), which can affect the absorption and metabolism of carbohydrates, fats, amino acids, and esters. cAMP can regulate the expression of nutrients related to nutrition utilization by activating protein kinase A (PKA) related signaling pathways, thereby regulating the metabolism and utilization of nutrients [8]. cAMP binds and allosterically activates the transcriptional regulator cAMP receptor protein (CRP) and participates in regulating nearly 50% of the genes and operons in bacteria in the form of CRP-cAMP complexes [9,10]. CRP forms an active conformation only when it binds to cAMP. Moreover, the concentration of CRP-cAMP complex and its mediated biological response were affected by the intracellular concentration of cAMP. It was also found that the *cpdA* gene encoded phosphodiesterase could hydrolyze cAMP to 5'-AMP in vitro and in vivo. Hence, the concentration of cAMP in cells is determined by cAMP phosphodiesterase [11]. Studies have shown that deletion of the *cyaA* gene in the highly virulent strain *Salmonella choleraesuis*X3246 resulted in the loss of pathogenicity but maintenance of immunogenicity [12]. Yu [13] reported that *cyaA* gene deletion significantly reduced the virulence of *Salmonella typhimurium* but did not completely eliminate its pathogenicity in animals, with the growth rate of the mutant strain significantly lower than that of its parent strain. In recent years, our group isolated and identified ExPEC (XJ10) from the lungs of sheep that have died of respiratory infection [14].

In this study, by constructing a *cyaA* gene deletion strain of XJ10, we analyzed the role of the *cyaA* gene in ExPEC XJ10 in the regulation of pulmonary infection in the mouse model *in vitro* and *in vivo*.

Materials and Methods

Ethical Statement

This trial was approved by the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (Approval no: A2019-157).

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains and plasmids used in this study have been listed *(Table 1)*. Wild-type (WT) ExPEC strain XJ10 was isolated from the lungs of diseased sheep. Plasmid pDS132 was used as a homologous recombination vector. *E. coli* β2155 was used as the transfer host for pDS132. *E. coli* DH5α, pDS132, and plasmid pHSG396 were purchased from Takara Bio (Otsu, Japan). The strains stored in the -80°C refrigerator were inoculated on MacConkey (Haibo Bio, Qingdao, China) agar plate and cultured at 37°C for 18 h [15], the single colonies were collected and incubated in LB liquid medium at 37°C for 12 h, the bacteria were collected to prepare for the next test.

Construction of *cyaA* Mutants and Complementary Strains

Gene mutants were constructed by homologous recombination [16]. Using the primer (Beijing RuiboXingke Biotechnology Co., Ltd., Beijing, China) sets *cyaA*-F1/R1 and *cyaA*-F2/R2, the upstream and downstream regions of *cyaA* gene were amplified separately by PCR from the genomic DNA of the WT strain, which was then mixed for overlapping PCR. The primer set *cyaA*-F1/R2 was added to amplify the target fragment. The plasmid pDS132-Δ*cyaA* containing the target fragment was introduced into *E. coli* β2155 [17], which was co-cultured with the WT strain. Colony PCR was performed for transformants resistant to sucrose but sensitive to chloramphenicol, and deletion of the *cyaA* gene was confirmed using the primers *cyaA*-outF/outR and *cyaA*-inF/inR. The final selected strain was named XJ10/Δ *cyaA*.

To construct a *cyaA* gene complementary strain, the complete *cyaA* gene sequence was amplified by PCR from the genomic DNA of the WT strain using the primer set HF1/HF2 and then cloned into the pHSG396 vector.

Table 1. *Strains and plasmids used in this study*

Strain Or Plasmid		Relevant characteristics	Reference
Strain	XJ10	Wild-type (WT), sheep origin, separation and preservation in the author's laboratory	[14]
	XJ10/Δ*cyaA*	Mutant deleted a 2457-bp fragment from whole ORF of *cyaA* in XJ10	This study
	cyaA$^+$/Δ*cyaA*	XJ10/Δ*cyaA* mutant complemented with a copy of the *cyaA* via pHSG396	This study
	E. coli β2155	Conjugated transfer host of pDS132	Laboratory
Plasmids	pDS132	Cm^R Sac B, suicide vector	Laboratory
	pDS132-Δ*cyaA*	pDS132 vector inserted disrupted *cyaA* in *SmaI*	This study
	pHSG396	ori lac Z CmR	Takara
	pHSG396-*cyaA*	pHSG396 vector containing the full-length *cyaA*	This study

Table 2. Specific primers for the construction of cyaA gene deletion strain of XJ10

Primers	5′-3′ Sequence	Application
*cyaA*1	TTGTACCTCTATATTGAGACTCTGAAAC	Mutant
*cyaA*2	TCACGAAAAATATTGCTGTAATAGCG	
cyaA-F1	*TCCCCCGGGGTCCCAGACCTTGCGGGAA*	Mutant
cyaA-R1	ATGGCATCCAGTCTCTGTTTCAGAG	
cyaA-F2	CTGCCAATCAGGATCACGATACG	Checking PCR
cyaA-R2	CCCCCGGGCTGGTAGTTTCGTGATCGGCAC	
cyaA-outF	CGTTCAGCCAGTTGGCACTG	Checking PCR
cyaA-outR	CTTTCTATCATCGCAGGAGAAGACG	
cyaA-inF	CGTTGACCTGCTGTACCGCAACT	Checking PCR
cyaA-inR	CAGTTGCTGCACGCGAGTACG	
HF1	CCGCTCGAGTTGTACCTCTATATTGAGACTCTGAAAC	Complementation
HF2	CCGGAATTCTCACGAAAAATATTGCTGTAATAGCG	
HJ1	AACCGCTTCCGTCATAAT	Checking PCR
HJ2	GGTTTACCAGCGTCACTTTA	

Plasmid pHSG396-*cyaA* was transformed into the *cyaA* gene mutant by electroporation. Primer set HJ1/HJ2 was used to re-acquire a complete clone of the *cyaA* gene. The complementary strain was named *cyaA*$^+$/Δ*cyaA*. The primer sequences used in this study has been shown *(Table 2)*.

Growth Analysis

A growth curve was plotted to determine the effects of the *cyaA* gene on the growth of ExPEC. 10^4 cfu mL^{-1} of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* strains were added to LB medium respectively and were inoculated at 37°C with 200 rpm oscillation. Samples were collected at 2, 4, 6, 8, 10, 12, 14, 16, and 18 h, placed in MacConkey solid medium, incubated at 37°C, and then counted separately. Data were obtained from three independent experiments, each in triplicate [18].

Analyses of Biochemical Characteristics and mRNA Levels of Various Genes Associated with Carbon Source Utilization, Adhesion, and Invasion

Use 0.45% NaCl solution to prepare bacterial suspensions from the single colonies of three bacteria cultured for 18 h, and set the concentration to 0.55 using a turbidimeter. Biochemical reaction characteristics of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* were analyzed by the biochemical analyzer (Vitek2 compact, Meyrié) (including 47 biochemical reaction items involving sugar decomposition, amino acid utilization, and enzyme production) [19]. According to the OMG kit (OMEGA Bio-tek, Inc., Norcross, GA, USA) instructions, the 3 strains were cultured at 37°C for 12 h and collected at 10^7 cfu mL^{-1} for RNA extraction. The RNA was later reverse-transcribed into cDNA using of Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The Roche real-time fluorescence, quantitative PCR system (Basel, Switzerland) was used to measure the mRNA levels of eight genes: the pathogenicity island gene *(fyuA)*, QS system gene *(luxS)*, P pili gene *(pap)*, flagellum gene *(motA)*, and genes associated with carbon source utilization *(sucC*, *acs*, *gltA*, and *fabB)*. The complete list of primers used in this study has been shown *(Table 3)*, and the MIQE guidelines [20] associated with them are shown in the supplementary materials.

Adhesion and Invasion Assay

The adhesion and invasion abilities of the three bacterial strains on mouse alveolar epithelial TC-1 cells (iCell Bioscience Inc, Shanghai, China) were determined. TC-1 cells were inoculated into 12-well tissue culture plates (NEST Biotechnology, Jiangsu, China) at approximately 10^5 cells per well. After the fusion degree of the cells reached 80%, TC-1 cells were infected at an MOI of 10. Low-glucose RMPI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) was added to every 1 mL of medium. The cells were incubated for 2 h at 37°C. After washing three times with PBS, the infected cells were lysed with 0.1% Triton X-100 (Solarbio, Beijing, China) for 3 min. Samples were diluted and plated on MacConkey agar plates to determine the number of bacterial (adherent cells and intracellular bacteria) colony-forming units (CFU). To measure the number of invading bacteria, the cells in the plates were washed with PBS after 2 h of incubation, and then further incubated with RMPI-1640 medium (include 25 μg mL^{-1} bleomycin, Thermo Fisher Scientific) for 90 min at 37°C to eliminate extracellular bacteria. Monolayer cells were washed with PBS, lysed with 0.1%

Table 3. *Primers used for real-time fluorescence quantitative amplification*		
Gene	**Primers**	**5′-3′ Sequence**
Adhesion-associated genes	*pap*1	TTTCTGCCTTACCTGTTTG
	*pap*2	TTTGCACCGTTCACCA
	*motA*1	TGCCGTTGCTGTTTCGTC
	*motA*2	TTCTGCCTCGCTTTCGTG
	*luxS*1	TGCGTGCCGAACAAAGAA
	*luxS*2	CAGCCCATTGGCGAGATA
	*fyuA*1	GCATCAGCCAACAACG
	*fyuA*2	CTCGCAGCAACTCCAC
Carbon source utilization related genes	*sucC*1	TTTGCCCGCTATGGCTTAC
	*sucC*2	CACGGATGTCTTCTTTGCTGTT
	*fbaB*1	CGCTAAGTTACCCGAATACC
	*fbaB*2	TGACTCTTCCGAGCCAAA
	*gltA*1	TCCTGCTGAATGGTGAAA
	*gltA*2	GGGTAAACAAATGGCTGA
	*acs*1	ACGCAGAAGTCCGCAACT
	*acs*2	TTCTCGACTACGCCAGGAT
Mouse lung tight junction and basement membrane gene	*occluding*1	GCTCTCTCAGCCAGCGTACT
	*occluding*2	GGCGATGCACATCACAATAA
	*mmp-9-*91	GCCAACTATGACCAGGAT
	*mmp-9-*92	TGCCACCAGGAACAGG
	*zo-1*1	AAAACCCGAAACTGAT
	*zo-1*2	CGCCCTTGGAATGTATG
Reference gene	*16SRNA*1	GAGCAAGCGGACCTCATA
	*16SRNA*2	ATTCACCGTGGCATTCTG
	*β-actin*1	TGCTATGTTGCTCTAGACTTCG
	*β-actin*2	GTTGGCATAGAGGTCTTTACGG

Table 4. *Biochemical reaction results*								
Parameter	**SAC**	**ADO**	**dMNE**	**dMAN**	**IARL**	**BGAL**	**dGLU**	**dSOR**
WT	+	+	+	+	-	+	+	+
XJ10/Δ*cyaA*	-	-	+	-	-	-	+	-
cyaA^{+}/Δ*cyaA*	-	+	+	+	-	+	+	-

Single colonies of the purified different strains were selected for biochemical identification
'+' indicates positive results; '-' indicates negative results
SAC: Saccharose; ADO: Adonitol; dMAN: D-maltose; BGA: β-galactosidase; dGLU: D-glucose; dSOR: D-Sorbitol; WT: wild type

Triton X-100, and plated to count invading bacteria. Each assay was performed in at least triplicate [21].

LD_{50} Determination

All animal experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (1985). The total bacterial dose required for each group of mice was calculated according to the ratio of concentration difference between the minimum dose of all dead mice and the maximum dose of all surviving mice according to the modified Karber's method [22] (doses are shown in *Table 4*). The mice provided by the Xinjiang Medical University laboratory animal center were randomly divided into six groups of six mice each. Mice were infected with bacteria by intraperitoneal injection. The mice were then provided with a clean environment, clean water, and adequate food. The mice were observed continuously for 7 days, and the

number of dead mice in each group was recorded. At the end of the experiment, the surviving mice were treated humanely and harmlessly.

Effect of *cyaA* on mRNA Levels of Genes Related to Mouse Lung Tight Junction and Basement Membrane Inflammation

The bacterial count of the three strains was adjusted to 2/3 of the LD_{50} of XJ10 and the strains were injected intraperitoneally into mice. At 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96, and 108 h after CO_2 asphyxiation, the lung tissues of mice were collected and the corpses were treated innocuously. The OMEGA kit (Omega Bio-tek, Inc., Norcross, GA, USA) was used to extract total RNA from the harvested tissue, followed by reverse transcription of RNA into cDNA using the Thermo Fisher Scientific reverse transcription kit. The mRNA levels of transmembrane protein *(occludin* gene), accessory protein *(zo-1* gene), and matrix metalloproteinase *(mmp-9* gene) were measured using the Roche real-time fluorescence, quantitative PCR system. The primer sequences used in this study have been shown (*Table 3*), and the MIQE guidelines [20] associated with them are shown in the supplementary materials.

Statistical Analysis

All results of data analysis are presented as the mean ± standard deviation (SD). Data analysis was performed on independent samples using the *t*-test. Differences were considered as significant when $P \leq 0.05$. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, Inc., La Jolla, CA, USA).

Results

Screening of Deletion Strain

The deletion strain was identified using primers *cyaA*-inF/inR. The amplified XJ10 produced a 193-bp band, whereas the deletion strain produced no bands *(Fig. 1-A)*. Using *cyaA*-outF/outR, the amplified XJ10 produced a 4139-bp band and the deletion strain produced a 1686-bp band *(Fig. 1-B)*. These results show that the deletion strain was successfully constructed.

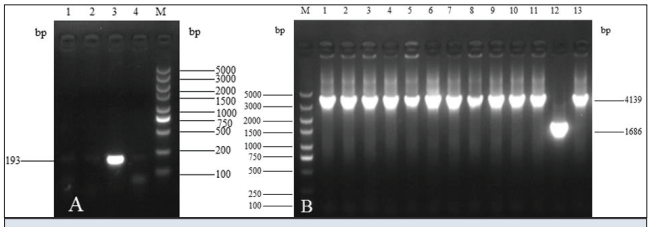

Fig 1. PCR results of homologous recombinant strains. A: (inF, inR) M: DL 5000 DNA Marker; 1,2,4: XJ10-Δ*cyaA*; 3: XJ10; B: (outF, outR) M: DL 5000 DNA Marker; 1-11,13: XJ10; 12: XJ10-Δ*cyaA*; The 3rd lane in A and 12th lane in B are the correct test results of the primers

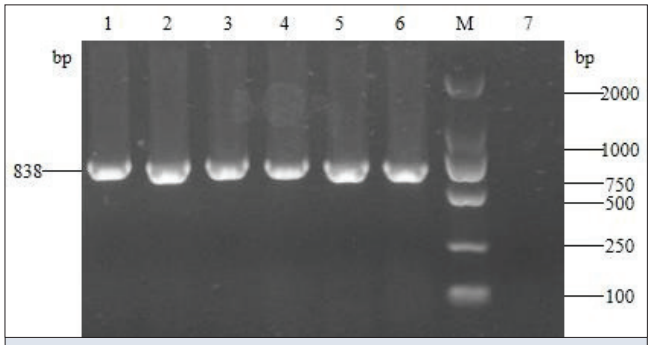

Fig 2. Complementary strain identification PCR results. 1-5: complementary strain; 6: positive control; 7: negative control; Using primers, complementary strains and WT showed amplified fragments of the same size

Screening Results of Complementary Strain

The full-length *cyaA* gene was amplified using HF1/2 and ligated with pHSG396 to construct the recombinant plasmid pHSG396-*cyaA*. The recombinant plasmid was transformed into XJ10-Δ*cyaA* via electroporation to construct *cyaA*$^+$/Δ*cyaA*. PCR identification was performed using primer HFJ1/2. Both XJ10 and *cyaA*$^+$/Δ*cyaA* amplified fragments 838 bp in size, whereas XJ10-Δ*cyaA* did not amplify any fragments *(Fig. 2)*, demonstrating successful construction of the complementary strain.

Growth Curve Measurement Results

The growth pattern of XJ10/Δ*cyaA* over 18 h was similar to that of XJ10 and *cyaA*$^+$/Δ*cyaA*, all of which reached a stable phase at 6 h. However, the number of live XJ10/Δ*cyaA* in the stable phase was significantly lower than that of XJ10 ($P<0.05$). The complementary strain *cyaA*$^+$/Δ*cyaA* did not reach the number of live XJ10 cells but was significantly higher than that of the gene deletion strain XJ10/Δ*cyaA* ($P<0.05$) *(Fig. 3)*.

Biochemical Identification and Analysis of mRNA Levels of Genes Related to Carbon Source Utilization, Adhesion, and Invasion

Biochemical identification of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* was conducted using a biochemical analyzer.

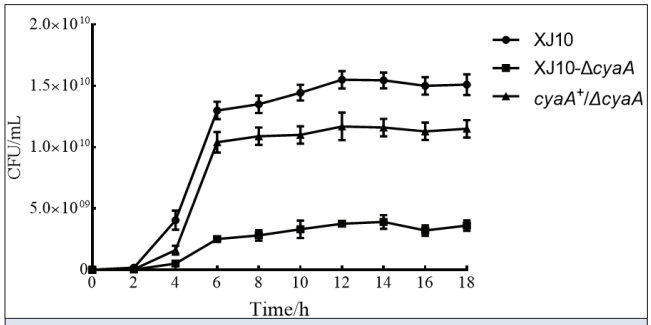

Fig 3. Growth curve. The WT strain (●), *cyaA* mutant (▪), and complemented strain (▲) were grown in LB medium at 37°C, respectively, and the number of live bacteria was measured every 2 h. The graph is representative of three independent experiments

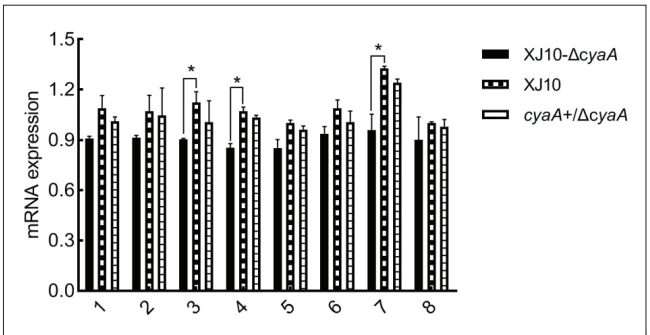

Fig 4. mRNA expression levels associated with carbon source utilization, adhesion, and invasion. (1: *sucC*; 2: *acs*; 3: *gltA*; 4:*fabB*; 5: *fyuA*; 6: *luxS*; 7: *pap*; 8:*motA*)

The mRNA expression levels of some genes related to carbon source utilization, adhesion, and invasion were determined. RNA extracted from the same WT, *cyaA* mutant, and complementary strain of CFU was reverse-transcribed into cDNA and subjected to real-time fluorescent quantitative PCR. All values are presented as the mean ± SD. Asterisks indicate significant differences between the mutants and WT strain (*P<0.05)

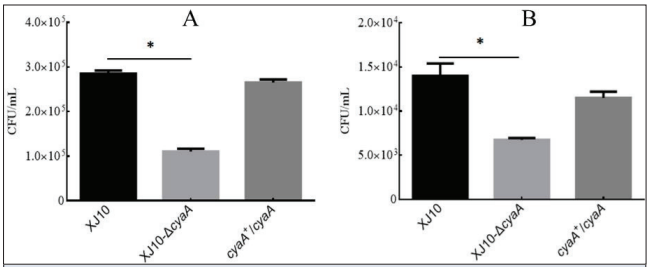

Fig 5. Effects of cyaA deletion on XJ10 adhesion and invasion mouse alveolar epithelial TC-1 cells. A: adhesion; B: invasion. Adhesion and invasion levels of ExPEC strains were measured. Cell associated bacteria (adherent + intracellular) were quantified after a 2 h infection period. Invasion was determined after bleomycin treatment for an additional 2 h. All values are presented as the mean ± SD. Asterisks indicate significant differences between the mutants and WT strain (*P<0.05)

The results are shown in *Table 4*. Compared to XJ10, XJ10/Δ*cyaA* lost the ability to utilize Saccharose (SAC), Adonitol (ADO), D-maltose (dMAN), β-galactosidase (BGAL), and D-Sorbitol (dSOR); *cyaA*$^+$/Δ*cyaA* regained the ability to utilize ADO, dMAN, and BGAL.

Compared to the WT strain, the mRNA expression levels of the eight genes in XJ10-Δ*cyaA* were all decreased. The expression levels of *gltA*, *pap*, and *fabB* were decreased

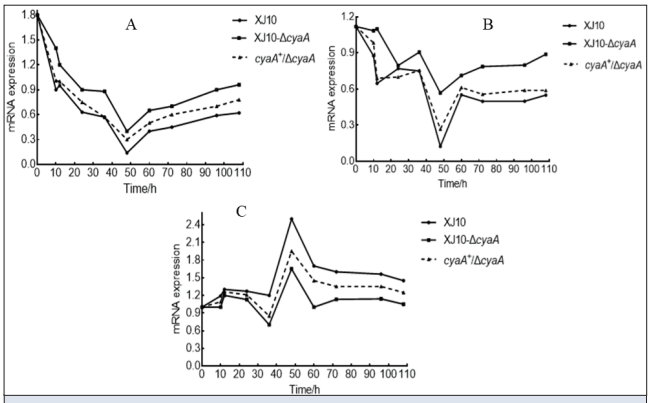

Fig 6. Effect of XJ10-*cyaA* on mouse lung tight junction and basement membrane-associated gene mRNA levels. A: *occludin*, B: *zo-1* (B), C: *mmp-9*. Animals were inoculated by intraperitoneal injection and lung samples were collected from the mice every 2 h. RNA was extracted from the samples and reverse-transcribed into cDNA for real-time PCR. All values are presented as the mean ± SD

significantly (P<0.05), whereas those of *fyuA*, *luxS*, *motA*, *sucC*, and *acs* were decreased but not significantly (P>0.05). Compared to XJ10, the mRNA expression levels of the eight genes in the complementary strain *cyaA*$^+$/Δ*cyaA* were all decreased, but the difference was not significant (P>0.05) *(Fig. 4)*.

Adhesion and Invasion Assay

Compared to XJ10, the adhesion and invasion abilities of XJ10-Δ*cyaA* for TC-1 cells were significantly lower (P<0.05) *(Fig. 5)*. The adhesion and invasion levels of XJ10-Δ*cyaA* for TC-1 cells were 2.58- and 2.07-fold lower those that of the WT strain, respectively. *cyaA*$^+$/Δ*cyaA* showed restoration of the adhesion and invasion abilities to some extent. These results demonstrate that the *cyaA* gene plays an important role in the cell adhesion and invasion abilities of ExPEC.

LD_{50} Measurement Results

The mouse infection model was used to determine the role of the *cyaA* gene in the virulence of ExPEC *in vivo*. Compared to LD_{50} of the WT ($10^{9.45}$ CFU) and *cyaA*$^+$/Δ*cyaA* ($10^{9.58}$ CFU) strains, that of the XJ10-Δ*cyaA* ($10^{9.71}$ CFU) strain was increased by 1.8- and 1.3-fold, respectively *(Table 5)*.

Table 5. *LD_{50} of XJ10, XJ10/ΔcyaA and cyaA$^+$/ΔcyaA*

Inoculation Dose	XJ10	XJ10-Δ*cyaA*	*cyaA*$^+$/Δ*cyaA*
$10^{10.08}$	6/6	6/6	6/6
$10^{9.82}$	6/6	5/6	5/6
$10^{9.56}$	4/6	1/6	3/6
$10^{9.30}$	1/6	0/6	1/6
$10^{9.04}$	1/6	0/6	0/6
$10^{8.78}$	0/6	0/6	0/6

Animals were inoculated by intraperitoneal injection and observed for 7 days. The ratio indicated the number of dead mice/ number of mice infected

Effect of *cyaA* on Some Genes Related to Mouse Lung Tight Junction and Basement Membrane Inflammation

The mRNA levels of *occludin* and *zo-1* in the three strains showed an initial decrease during the test period, reaching minimum values at 48 h, and then increasing until 108 h. However, the mRNA expression levels of *occludin* and *zo-1* in XJ10-Δ*cyaA* were always significantly higher than those of XJ10 and *cyaA*$^+$/Δ*cyaA* throughout the experiment (P<0.05) *(Fig. 6-A,B)*. The mRNA levels of *mmp-9* for all three strains increased from 0 to 48 h, and reached their peak at approximately 48 h, after which the levels began decreasing until 108 h. However, the *mmp-9* mRNA level of XJ10-*ΔcyaA* was significantly lower than that of XJ10 and *cyaA*$^+$/Δ*cyaA* throughout the experiment (P<0.05) *(Fig. 6-C)*.

Discussion

ExPEC is capable of causing infections in many extra-intestinal systems, including the urinary tract, central nervous system, circulatory system, and respiratory system. This is caused by the joint action of the virulence factors carried by ExPEC, which not only enhances the virulence of ExPEC but also extraintestinal infection of the host [23,24]. In this study, we first evaluated the effect of the *cyaA* gene on ExPEC virulence using the sheep-derived clinical strain XJ10, deletion strain, and complementary strain. The *cyaA* gene deletion strain showed a lower growth rate than the WT and complementary strains, as well as defects in carbon source utilization, cell adhesion, and invasion. The LD_{50} indicated that the loss of gene attenuated the virulence of ExPEC in mice.

Liao [25] found that the *cya* gene mutant of *S. typhimurium* SL1344 lost its ability to utilize glycerol, maltose, and sorbitol. Wang [26] showed that the growth rate of the *cya* gene mutant of *E. coli* G8107 in goose yolk peritonitis was slower at each stage than that of the corresponding parent strain. The results of this study demonstrate that the growth rate of the *cyaA* gene mutant was significantly lower than that of WT and complementary strains. The *cyaA* gene deletion strain lost its ability to utilize some carbon sources, and the mRNA expression of genes related to sugar utilization was significantly decreased. These results suggest that the deletion of the *cyaA* gene led to a loss of efficiency in AC synthesis, resulting in the inability to catalyze the conversion of ATP into cAMP, in turn preventing activation of the receptor protein CRP. This further illustrates that the *cyaA* gene can regulate certain metabolic reactions in *E. coli*, thereby indirectly affecting its virulence. In addition, the experimental results showed that the *cyaA* gene knockout caused 4 genes related to carbon utilization to be down regulated, the growth curve of the bacteria also confirmed this result. Therefore, it is speculated that the deletion of the *cyaA* gene will affect the carbon source utilization of XJ10, thereby indirectly affect the growth of bacteria.

Liu [27] found that knocking out the *cya* gene in the *S. typhimurium* SL1344 strain resulted in decreased expression of genes related to the pili and cellulose as well as significantly reduced adhesion to and invasion of HeLa cells, whereas the deletion strain showed lower virulence for HeLa cells. In this study, deletion of *cyaA* gene significantly reduced the adhesion and invasion ability of XJ10 for mouse alveolar epithelial cells TC-1, and the mRNA levels of genes associated with adhesion and invasion were decreased significantly, whereas the complementary strain showed restored adhesion and invasion abilities to a certain extent. This supports that *cyaA* gene can regulate the expression of genes related to the flagellum, pili, or a series of surface proteins to affect the target cells, thereby reducing the virulence of XJ10. Lung tight junctions are composed of Occludins and accessory proteins (ZO-1). When these proteins are altered, the permeability of the intercellular space increases, causing tumor metastasis, destruction of organ physiological barriers, the spread of inflammation [28], etc. MMP-9 belongs to a group of matrix metalloproteinases expressed in a variety of cells and involved in physiological and pathological processes such as cell movement, inflammation, and tissue repair [29]. Jin found that the protein expression of ZO-1 in alveolar epithelial cells from mice was decreased following acute lung injury [30]. This study showed that the mRNA levels of the *occludin* gene and *zo-1* gene in the lung tissue of mice artificially infected with XJ10/Δ*cyaA* were significantly increased, whereas the expression of the inflammation-related *mmp-9* gene was decreased. The *cyaA* gene may be associated with XJ10 causing lung tissue pathological changes.

By reducing the utilization of certain nutrients such as SAC, ADO, dMAN, BGAL, and dSOR, deletion of the *cyaA* gene can reduce the growth rate of XJ10, affect its adhesion and invasion abilities in sensitive cells, and influence certain factors related to cell barrier and inflammation in the lung tissue, thereby decreasing the virulence of XJ10. However, the complementary strain cannot utilize D-sorbitol and sucrose, possibly because the construction of the complementary strain only expresses the *cyaA* gene in the bacterium through plasmid expression, without reintegrating the *cyaA* gene into the bacterial genome. Therefore, the relevant biological characteristics of the complementary strains have not fully recovered to be consistent with those of the wild strains. Based on these results, the LD_{50} of the bacteria in mice was determined. The results showed that the LD_{50} of XJ10 with *cyaA* gene deletion was 1.8-fold higher than that of WT, and its virulence was significantly reduced. The construction of

the XJ10/Δ*cyaA* mutant strain in this experiment provides a basis for comparing the virulence intensity of the XJ10/Δ*cyaA* mutant and wild strains, conducting functional analysis of the *cyaA* gene, and exploring novel approaches for developing attenuated live vaccines against *E. coli*.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author/s upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

This trial was approved by the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (Approval no: A2019-157).

Author Contributions

Conceived of or designed study: X Z, ML H, X H; Performed research: YJ C, XX G; Analyzed data: FG Z, J L; Contributed new methods or models: TZ W, XX Z; Wrote the paper: YJ C, YN S, XL W.

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Research Article

Overview on Antioxidant and Oxidative Stress Markers after Garlic Oil Supplement in Suckling Buffalo Calves

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Abstract

Calves during the suckling period have serious attention and induce immense bearing on early maturity and production. This work aimed to scrutinize the affections of garlic oil supplements on antioxidants, oxidative stress, and immune response in suckling buffalo calves. To achieve this aim we used twenty suckling buffalo calves, and divided them into two groups. The first group (n=10) did not receive any supplement, and the second group (n=10) received garlic oil as a supplement 5 mL/calf/day orally for 7 days. Whole blood and serum samples were collected on the 1st, 3rd, 5th, and 7th day of supplement from all calves. Our data showed that the supplemented garlic oil significantly increase lysated blood and serum SOD, CAT, GSH, and TAC levels, while a gradual decrease in MDA and non-significant changes in NO as indicators of oxidative stress, associated with a significant increase in serum vitamins A and E, selenium, iron, and zinc. Total protein, albumin, globulin, γ1, γ2, α1, α2 were gradually and significantly increased in comparison with the non-supplemented control group. In conclusion, garlic oil supplement have promotives effects on enzymatic and non-enzymatic antioxidant system, immunity, nutrient utilization required for immunity modulation and regulation in calves.

Keywords: Antioxidant, Buffalo calves, Electrophoresis, Garlic oil, Oxidative stress, Vitamin

Introduction

Calves were considered a fundamental stage for livestock farming. As calf rearing is essential to the continued existence of the dairy industry, it is also important to obtain and conserve good quality genetic material. Calf rearing depends on good hygiene management and good nutrition [1]. For calves to grow well, it is very important to get good nutrients, probiotics and feed supplements. Supplementing with rumen modulators, liver tonics, and immunomodulators at a young age helps boost immunity and prevent disease [2,3].

Most of the substances required for a calf feed to achieve promising results have been found in herbs. The beneficial effects of herbs on farm animals include activation of feed intake, stimulate the immune system, as well as antibacterial, anthelmintic, antiviral, anti-inflammatory activity, and antioxidant effects. Herbs also meet the animal's nutritional needs, stimulate the endocrine system and the metabolism of intermediate nutrients [4]. Garlic contains many nutrients and active substances such as organo-sulphur compounds, proteins, free amino acids, vitamins, trace elements, enzymes, flavonoids, phenols and organo-selenium compounds [5,6].

Garlic supplementation through feed has many favorable experimental and clinical benefits, which include stimulation of immune function, improved detoxification of foreign substances, and restoration of physical strength and resistance to various stresses. Allicin is the main bioactive component of garlic and may be responsible for some effects of garlic [7]. The flavonoids contained in garlic oil interact with radical stabilizing of reactive oxygen species (ROS). The radicals were inactivated by the strong reactivity of the hydroxyl group on the flavonoids [8,9]. The antioxidant properties of garlic oil and its constituent diallyl disulfide have been reported. They owe their antioxidant effect to organo-sulphur compounds [10]. Feed additives containing garlic provided calves with higher

serum total protein and globulin levels, as well as improved liver function with better digestibility of nutrient leading to good calf performance [11].

Therefore, this study was designed to scrutinize the effects of garlic oil supplements on antioxidants, oxidative stress, immune response, and performance in suckling buffalo calves.

Material and Methods

Ethical Statement

This protocol was approved by the Research Committee of the Animal Health Research Institute and authorized by The Institutional Animal Care and Use Committee (ARC-IACUC)/Agricultural Research Center (ARC/AHRI/43/23).

Experimental Design

This work was established on a private farm of buffalo. Twenty suckling buffalo calves aged (15-21) days and weighted (42±5) kg, were used and divided into two groups (n=10). The first group (G1) calves did not receive any supplement, used as a control group. The second group (G2) calves received garlic oil supplements in a dose of 5 mL/calf/day orally after 1 h post morning breastfeeding for 7 days continuously. All calves received breastfeeding twice daily with a little of roughage, concentrate diets and water ad-libitum.

Sampling

A sample of the garlic oil was sent to lab for gas chromatographic analysis. Blood samples were collected from the jugular vein of all calves on the 1st, 3rd, 5th, and 7th days of garlic oil supplement. Each blood sample was collected in two tubes, the first tube with EDTA as anticoagulant for whole blood obtaining and used to prepare the RBCs lysate, and the second one was collected without anticoagulant for serum obtaining. Serum samples were obtained by centrifuging the blood samples at 3000 rpm for 5 min. The clear sera were transferred into clean dry eppendorf tubes and stored at -20°C till biochemical analysis.

Chemicals and Materials

Garlic oil; GARLIC OIL HUILE DE AIL, manufactured by El Captain Company (CAP Pharm) for extracting natural oils, plants and cosmotics.

Drabkin′s reagent; Contains sodium bicarbonate, potassium ferricyanide, and potassium cyanide, Catalog Number D5941, SIGMA-ALDRICH, St. Louis, MO 63103, USA.

Gas Chromatography Examination of Garlic Oil

The chemical composition of the samples was determined using a GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a TG–5MS direct capillary column (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was first maintained at 60°C, then increased by 5°C/min to 250°C for 2 min then increased to 300 at 30°C/min. The injector temperature was maintained at 270°C. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The solvent delay was 4 min and 1 µL of diluted samples was injected automatically using auto sampler AS3000 coupled to the GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source and transfer line were set at 200°C and 280°C respectively. The components were identified by comparing their mass spectra with those of WILEY 09 and NIST14 mass spectral database, according to the method identified by Kareem et al.[12].

Hematology and Blood Redox Parameter Analysis

Whole blood samples were used for detection of RBCs antioxidant parameters. Preparation of lysate and assays of antioxidant parameters, RBCs were separated from plasma by centrifugation, washed three times with saline and lysed. The lysate was mixed with an equal volume of Drabkin′s reagent to determine the hemoglobin levels [13]. Catalase activity (CAT), malondialdehyde (MDA) as indicator of lipid peroxidation and reduced glutathione (GSH) in lysated RBCs were determined according to Aebi [14], Okhawa et al.[15] and Pleban et al.[16], respectively. Superoxide dismutase (SOD) were estimated according to Nishikimi et al.[17].

Serum Biochemical Analysis

Serum zinc (Zn), iron (Fe) and selenium (Se) levels were detected by using atomic absorption Spectrophotometer (A7942, SensAA-Dual, GBC Scientific Equipment, Braeside, Victoria, Australia), retinol (vitamin A) and α-tocopherol (vitamin E) concentrations in serum determination were performed according to the method described by Roberts et al.[18]. Total antioxidant capacity (TAC) were estimated by using Bio-diagnostic kit CAT. No. TA2513 and nitric oxide (NO) was determined by kits CAT. No. NO 25 33.

Total Protein and Electrophoretic Protein Estimation

Total protein and electrophoretic pattern were estimated according to Sonnenwirth et al.[19] and Davis [20], respectively, and calculated according to SynGene S. No. 17292*14518 sme*mpcs.

Statistical Analysis

Statistical analysis of data using an Excel T.test to test the significance difference between the two groups. Results are presented as means ± SE, those considered statistically significant at $P<0.05$, highly significant at $P<0.01$, and very highly significant at $P<0.001$.

Results

The gas chromatographic analysis of garlic oil results *(Table 1; Fig. 1)*, revealed a constituent of fatty acids such as Hexadecanoic Acid (Palmitic Acid), 9,12-Octadecadienoic acid, Erucic Acid (13-Docosenoic Acid) and Cis-13-Octadecenoic Acid (Oleic Acid). Organosulphur compounds such as Di-2-Benzothiazole Disulfane, 2-Aminoethanethiol Hydrogen Sulfate, 3-Allyl-2-Methylthio-4-Phenylthiophene. Flavonoids and phenolic derivatives such as 03027205002 Flavone 4'-OH, 5-OH, 7-Di-O-Glucoside, 4H-1-Benzopyran-4-One, 2-(3,4-Dimethoxyphenyl)-3,5-Dihydroxy-7-Methoxy, 4H-1-Benzopyran-4-One, 2-(3,4-Dihydroxyphenyl)-6,8- Di-Á-D-Glucopyranosyl-5,7- Dihydroxy.

The results of antioxidants and oxidative stress in lysated blood and serum in calves received garlic oil supplement *(Table 2)* revealed a gradual increase of SOD, CAT, and

Table 1. *Gas chromatography-mass spectrometry (GC-MS) analysis of garlic oil*

RT	Compound Name	Molecular Formula	Molecular Weight
4.03	Di-2-Benzothiazole Disulfane	C14H8N2S4	332
7.06	5à-Androstan-16-one, cyclic ethylene mercaptole	C21H34S2	350
9.51	2-Aminoethanethiol Hydrogen Sulfate (Ester)	C2H7NO3S2	157
11.14	Ethanimidothioic Acid, 2-(Dimethylamino)-N-[[(Meth Ylamino)Carbonyl]Oxy]-2-O Xo-, Methyl Ester	C7H13N3O3S	219
13.66	2-Myristynoyl pantetheine	C25H44N2O5S	484
15.76	tert-Hexadecanethiol	C16H34S	258
18.23	3-Methyl-4-(Phenylthio)-2-Prop-2-Eny L-2,5-Dihydrothiophene 1,1-Dioxide	C14H16O2S2	280
21.75	2-Methylthio-3,4-Dihydrona Phtho[2,1-C]Thiophene	C13H12S2	232
24.46	3-Benzylidene-2-(P-Methylp Henyl)Sulfonylamino-1-Me Thylindoline	C23H20N2O2S	388
25.8	3-Allyl-2-Methylthio-4-Phenylthiophene	C14H14S2	246
26.52	Hexadecanoic Acid (Palmitic Acid)	C16H32O2	256
28.26	1-Methoxy-3-Pentyl-6,6a,7,8-Tetrahydro-6,6-Dimethyl-9 H-Dibenzo[B,D]Pyran-9-One (Pyrethrin 1)	C21H28O3	328
	Hexadecanoic Acid, Trimethylsilyl Ester (Palmitic Acid, Tms Derivative)	C19H40O2Si	328
29.74	11,14-Eicosadienoic Acid, Methyl Ester	C21H38O2	322
	9,12-Octadecadienoic Acid (Z,Z)-	C18H32O2	280
	Z-(13,14-Epoxy) Tetradec-11-En-1-Ol Acetate	C16H28O3	268
	1,2-15,16-Diepoxyhexadecane	C16H30O2	254
31.23	Cis-13-Octadecenoic Acid (Oleic Acid),(9-Octadecenoic Acid, (E),(Trans-13-Octadecenoic Acid),(Cis-Vaccenic Acid)	C18H34O2	282
33.07	Octadecanoic Acid	C18H36O2	284
35.0	Cis-11-Eicosenoic Acid	C20H38O2	310
	14-Á-H-Pregna	C21H36	288
	22-Tricosenoic Acid	C23H44O2	352
38.05	Tetrapentacontane, 1,54-Dibromo-(1,54-Dibromotetrapentacon Tane)	C54H108Br2	914
	17-Pentatriacontene	C35H70	490
	Heptacosane	C27H56	380
40.52	Erucic Acid (13-Docosenoic Acid)	C22H42O2	338
42.83	4H-1-Benzopyran-4-One, 2-(3,4-Dihydroxyphenyl)-6,8- Di-Á-D-Glucopyranosyl-5,7- Dihydroxy	C27H30O16	610
44.84	03027205002 Flavone 4'-Oh,5-Oh,7-Di-O-Glucoside	C27H30O15	594
	9,12-Octadecadienoic Acid (Z,Z)-, 2,3-Bis[(Trimethylsilyl)Oxy-Propyl Ester	C27H54O4Si2	498
	9,12,15-Octadecatrienoic Acid, 2,3-Bis[(Trimethylsilyl)Oxy-Propyl Ester, (Z,Z,Z)-	C27H52O4Si2	496
	Rhodopsin (.PSI.,.PSI.-Carotene, 1,2-Dihydro-1-Hydroxy)	C40H58O	554
45.11	4h-1-Benzopyran-4-One, 2-(3,4-Dimethoxyphenyl)-3,5- Dihydroxy-7-Methoxy	C18H16O7	344

RT: *Retention Time*

Table 2. *Results of antioxidant and oxidative stress, vitamins, and trace elements in suckling calves supplied by garlic oil (G2) compared with the control group (G1)*

Parameters	Time	Groups		
		G1	G2	P Value
SOD (U/g Hb)	1st day	5.214±0.216	5.234±0.204	0.884
	3rd day	5.376±0.201	5.602±0.237	0.143
	5th day	5.588±0.209	6.216±0.21 **	0.0015
	7th day	5.754±0.216	6.658±0.209 ***	0.00015
CAT (U/g Hb)	1st day	26.866±2.36	27.34±2.22	0.752
	3rd day	26.766±2.29	29.206±2.2	0.124
	5th day	26.584±2.275	30.858±1.678 **	0.0096
	7th day	26.078±1.843	33.174±1.613 ***	0.00019
TAC (mU/L)	1st day	1.144±0.136	1.184±0.141	0.66
	3rd day	1.216±0.144	1.392±0.138	0.084
	5th day	1.332±0.132	1.708±0.141 **	0.0025
	7th day	1.618±0.135	2.074±0.133 ***	0.00066
GSH (mmol/g Hb)	1st day	35.692±2.092	35.812±2.071	0.93
	3rd day	35.538±2.147	36.056±2.118	0.711
	5th day	35.502±2.074	37.266±2.092	0.217
	7th day	35.492±2.058	38.78±2.02 *	0.034
MDA (nmol/g Hb)	1st day	8.798±1.189	8.758±1.15	0.958
	3rd day	8.902±1.261	7.778±1.117	0.174
	5th day	9.052±1.282	6.938±0.824 *	0.015
	7th day	9.12±1.28	6.26±0.605 **	0.002
Nitric Oxide (NO) (nmol/L)	1st day	33.068±3.144	32.97±3.278	0.963
	3rd day	34.488±3.03	33.788±3.233	0.733
	5th day	35.08±3.011	34.482±3.223	0.7695
	7th day	36.142±3.023	35.228±3.083	0.6486
Vitamin E (µg/dL)	1st day	89.052±4.336	91.628±5.085	0.4138
	3rd day	91.528±4.904	105.34±6.016 **	0.0041
	5th day	96.17±5.938	119.64±7.062 ***	0.00046
	7th day	102.34±4.8	146.14±8.543 ***	0.0000085
Vitamin A (µg/dL)	1st day	54.824±3.894	56.036±3.449	0.61648
	3rd day	56.672±3.462	69.214±3.986 ***	0.000718
	5th day	61.316±3.933	82.928±4.903 ***	0.000058
	7th day	67.26±4.145	104.44±6.117 ***	0.0000035
Selenium (Se) (µg/dL)	1st day	6.322±0.334	6.448±0.368	0.58636
	3rd day	6.474±0.395	7.232±0.416 *	0.01832
	5th day	6.698±0.402	8.106±0.476 ***	0.000981
	7th day	7.052±0.368	9.762±0.565 ***	0.0000186
Iron (Fe) (µg/dL)	1st day	160.8±11.3	161.4±10.57	0.933
	3rd day	163.4±10.69	170.6±10.31	0.31
	5th day	172.4±10.67	181.8±10.31	0.194
	7th day	182.4±10.88	198.6±10.5 *	0.043
Zinc (Zn) (µg/dL)	1st day	156.14±10.97	157.28±10.04	0.868
	3rd day	159.32±10.48	166.68±10.1	0.291
	5th day	168.94±10.46	178.82±10.57	0.176
	7th day	179.48±10.71	196.5±10.37 *	0.034

** Superscript means significant at P<0.05; ** Superscript means highly significant at P<0.01; *** Superscript means very highly significant at P<0.001*

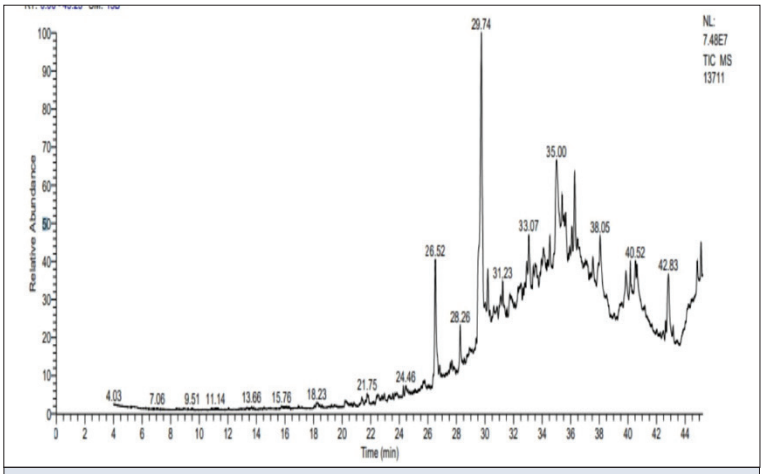

Fig 1. Curve of gas chromatography-mass spectrometry (GC-MS) analysis of garlic oil

Table 3. *Results of total protein, albumin, and globulin and protein electrophoresis in suckling calves supplied by garlic oil (G2) compared with the control group (G1)*

Parameters	Time	Groups		
		G1	G2	P Value
Total Protein (g/dL)	1st day	5.952±0.212	5.984±0.218	0.82
	3rd day	6.078±0.228	6.27±0.204	0.198
	5th day	6.224±0.231	6.604±0.212 *	0.027
	7th day	6.352±0.216	7.09±0.233 ***	0.00083
Albumin (g/dL)	1st day	1.982±0.071	1.975±0.072	0.875
	3rd day	2.006±0.075	2.075±0.067	0.162
	5th day	2.085±0.077	2.16±0.069	0.147
	7th day	2.128±0.072	2.34±0.077 **	0.002
Globulin (g/dL)	1st day	3.97±0.142	4.009±0.146	0.677
	3rd day	4.072±0.153	4.195±0.136	0.218
	5th day	4.139±0.153	4.444±0.142 *	0.011
	7th day	4.224±0.144	4.75±0.156 ***	0.0005
Alpha 1 (α1) (g/dL)	1st day	0.655±0.023	0.646±0.024	0.584
	3rd day	0.638±0.024	0.677±0.022 *	0.028
	5th day	0.672±0.025	0.726±0.023 **	0.007
	7th day	0.654±0.022	0.723±0.024 **	0.0015
Alpha 2 (α2) (g/dL)	1st day	0.548±0.02	0.562±0.02	0.272
	3rd day	0.577±0.022	0.589±0.019	0.382
	5th day	0.56±0.021	0.627±0.02 ***	0.0008
	7th day	0.597±0.02	0.681±0.022 ***	0.0003
Beta 1 (β1) (g/dL)	1st day	0.625±0.022	0.652±0.024	0.098
	3rd day	0.669±0.025	0.69±0.022	0.198
	5th day	0.685±0.025	0.66±0.02	0.14
	7th day	0.692±0.024	0.702±0.023	0.536
Beta 2 (β2) (g/dL)	1st day	0.565±0.02	0.562±0.02	0.825
	3rd day	0.577±0.022	0.577±0.019	0.966
	5th day	0.573±0.021	0.594±0.019	0.127
	7th day	0.597±0.02	0.61±0.02	0.351
Gamma 1 (γ1) (g/dL)	1st day	1.071±0.038	1.065±0.039	0.805
	3rd day	1.064±0.04	1.129±0.037 *	0.03
	5th day	1.12±0.042	1.242±0.04 **	0.002
	7th day	1.131±0.038	1.347±0.044 ***	0.00003
Gamma 2 (γ2) (g/dL)	1st day	0.506±0.018	0.521±0.019	0.245
	3rd day	0.547±0.021	0.533±0.017	0.275
	5th day	0.529±0.02	0.594±0.019 ***	0.0007
	7th day	0.553±0.019	0.688±0.023 ***	0.000007

** Superscript means significant at P<0.05; ** Superscript means highly significant at P<0.01; *** Superscript means very highly significant at P<0.001*

TAC from the 3rd day of supplement with a significant (P<0.01) increase on the 5th day and became significantly increase (P<0.001) on the 7th day. Also, there was a significant increase (P<0.05) of GSH on the 7th day with a gradual decrease of MDA until became significantly (P<0.05) decreased on the 5th day and the 7th day. There was a non-significant changes in nitric oxide (NO) in comparison with the control non-supplemented group.

Serum vitamin results *(Table 2)* revealed a significant increase (P<0.01) of vitamin E on the 3rd day with a gradual significant increase (P<0.001) from the 5th day of the supplement. Also, there was a gradual significant increase (P<0.001) of vitamin A from the 3rd day of the calves group that received garlic oil compared to the control non-supplemented group.

Trace element results in the serum of calves received garlic oil supplement *(Table 2)* revealed a gradual significant (P<0.05) increase of selenium (Se) from the 3rd day and extended gradually to be more significant (P<0.001) from the 5th day of supplement. Also, there was a gradual increase in iron (Fe) and zinc (Zn) levels till became a significant increase (P<0.05) on the 7th day of the supplement compared to the control non-supplemented group.

The results of serum total protein and its fractionations in calves received garlic oil supplement *(Table 3)* revealed a significant increase (P<0.05) on the 5th day and (P<0.001) on the 7th day of total protein and globulin, and a significant increase (P<0.01) on the 7th day of serum albumin level. On the other hand, there was a significant increase (P<0.05) of both gamma 1 (γ1) and alpha 1 (α1) globulin on the 3rd day and a gradual significant increase (P<0.01) from the 5th day. Both gamma 2 (γ2) and alpha 2 (α2) globulin were gradually significantly increased (P<0.001) from the 5th day of supplement, with non-significant changes of both beta 1 (β1) and beta 2 (β2) globulin compared to control non-supplemented group.

Discussion

The result of garlic oil gas chromatographically analysis that contains fatty acids, organosulphur compounds, flavonoids, phenolic derivatives and antioxidants bio-active compound were in accordance with the results recorded in previous studies [21,22]. Active components of garlic oil, diallyl sulphide (DAS) and diallyl disulphide (DADS), have been found to protect and treat oxidative damage [23]. Hexadecanoic acid constituents in garlic oil has immunomodulation effects [24]. Organosulphur compounds 2-Aminoethanethiol Hydrogen Sulfate (Ester), DAS, DADS and diallyl trisulfide which modulate the activity of several metabolizing enzymes that activate cytochrome or detoxify glutathione S-transferases and inhibit the formation of DNA adducts in several target tissues [25].

The antioxidant results of garlic oil-supplemented calves revealed an increase of SOD, CAT, TAC, and GSH, while decreased the oxidative stress marker MDA following the results recorded by Chen et al.[26] in rats and Alagawany et al.[27] in rabbits. These results also resemble the result recorded by Asghari et al.[4] by using herbal essential oil in suckling calves. These results may be attributed to the ability of garlic oil to improve antioxidant enzyme activity [28]. In the present study, the decreased MDA level in RBCs by garlic oil may be originated from the elevation of SOD, CAT, TAC, and GSH and/or its act as a radical scavenger. Garlic functions as an antioxidant by several mechanisms, and one of the best defense mechanisms against oxidative damage is enhanced by the antioxidant bioactive compound constituting in garlic. Garlic oil has a role as an antioxidant activator and decreases oxidative stress [29,30]. Another mechanism for the antioxidant activity of garlic is the reaction with free radicals, flavonoids constituents of garlic oil may prevent free radicals injury by a direct scavenging of free radicals. Flavonoids interact with the radical stabilizing of ROS. Radicals were inactivated by the high reactivity of the hydroxyl group on flavonoids [8,9]. Also, the activities of organosulphur compounds diallyl sulfide (DAS), and diallyl disulfide (DADS) constituted in garlic oil protect and treat oxidative damage [23].

The result of elevated serum levels of vitamins A and E with trace elements of selenium, iron, and zinc of garlic oil-supplemented calves may be attributed to the constituent of garlic oil to some active compounds, like that organosulphur compounds, enzymes, sterols, steroids, and organo-selenium compounds [5], also garlic containing many nutrient components such as protein, free amino acids, vitamins, and trace elements [6]. Garlic physiological effects are provided by organosulphur-containing compounds as well as flavonoids, minerals (Ca, Fe, K, Mg, Na, Zn), and vitamins (A, E, C, and B complex) [31].

The elevation of serum total protein, albumin, and globulin after garlic oil supplement in suckling calves was in agreement with Hassan and Abdel-Raheem [11] and Duvvu et al.[32], and similar results were recorded by Alagawany et al.[27] after garlic dietary supplement in growing rabbit, and El-Nomeary et al.[33] since supply essential garlic oil in the rabbit. These results provided the improvement of the metabolic and general health status of calves and the role of garlic in improving nutrient digestibility and protein utilization [11,32,34]. Garlic and its bioactive compounds activate protein utilization [35]. The albumin concentration involves the transport of several exogenous chemical compounds and endogenous metabolites and regulates osmotic pressure, whereas globulin is an important part of

the immune system [36]. Increased globulin levels indicated improved immune response. These results may be referred to the organosulphur compounds constitute in garlic and their immunomodulation effects [24,35]. This increase in globulin may be related to the elevation of gamma globulin in those following El-Nomeary et al.[33] in rabbits. These results provided the role of garlic oil supplements in improving the immunity and utilization of nutrients responsible for immunity modulation and regulation [5,27].

In conclusion, garlic oil supplement in suckling buffalo calves have a promotives effects on antioxidants, they can directly and rapidly scavenge free radicals and/or block their formation by increasing endogenous antioxidant and serum vitamins level. Garlic oil constitutes a bioactive compound that provided physiological effects, increasing immunity and helping in the utilization of nutrients responsible for immunity modulation and regulation, and by extension improving general health status of calves. So we recommend garlic oil be used as a supplement for suckling buffalo calves to increase the productivity of large animal farms.

Availability of Data and Materials

The data sets analyzed during the current study are available from the corresponding author (H. M. Yousif) on reasonable request.

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Ethical Statement

This protocol was approved by the Research Committee of the Animal Health Research Institute and authorized by The Institutional Animal Care and Use Committee (ARC-IACUC)/ Agricultural Research Center (ARC/AHRI/43/23).

Conflict of Interest

No potential conflict of interest was reported by the authors. Authors only are responsible for the content and writing of the paper.

Author Contributions

H. M. Yousif designed the research and collect the samples. M. K. Mansour, H. M. Yousif, A. M. El Mahdy and M. F. Hassan performed the experimental duties of this study and analyzed the data. M. K. Mansour and H. M. Yousif did the statistical analyses. All authors participate in writing and approved the final version of the manuscript.

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Research Article

Anterior and Posterior Segment Parameters of the Eye in Eagle Owls (*Bubo bubo*) [1]

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Abstract

Ocular anatomy may differ between species. In study design, it was aimed to create reference values by evaluating the anatomical formations of adult eagle owls with healthy eyes. The study materials consisted of 12 healthy eyes of 6 owl eagle birds brought to our hospital for non-ocular problems. The anterior and posterior eye segments were evaluated with the biomicroscopic examination, Schirmer tear test, color fundus photography, orbital ultrasonography, optical biometry, and keratometry without anesthesia. Anterior chamber deep (ACD), horizontal visible iris diameter (HVID), pupil diameter (PD), and base curve were topographically measured. Axial globe length (AGL), central corneal thickness (CCT), and lens power (LP) were measured. The optic disc, tapetal, non-tapetal region, retina, and choroid were evaluated with fundoscopic examination. Intraocular pressure (IOP) values were also recorded as average values and standard error. ACD 3.21±0.01 mm, HVID 9.02±0.004 mm, PD 7.97±0.10 mm, AGL 31.108±0.1773 mm, CCT 207.83± 0.50 µm, LP 16.5 Dioptri, IOP 13.817±0.2 mmHg, horizontal corneal diameter (HCD) 13.57±0.09 mm, a vertical corneal diameter (VCD) 13.20± 0.14 mm and the mean of basic curve (BC) was found as 10.93±0.10 mm. In conclusion, it is thought that it will be possible to detect more easily pathological conditions of eagle owl birds brought to veterinary clinics with eye or vision problems with reference values presented in this study, and the data obtained from the study will contribute to clinical practice.

Keywords: *Eagle owl, Eye anatomy, Ocular examination, Bubo bubo*

Introduction

All wild animals, especially the endangered ones, should be protected to preserve and maintain the natural balance. Predatory birds are at the top of the food pyramid. Therefore, their numbers are less than other living beings. These birds have to hunt for survival [1,2]. The eye is the most important weapon for both hunting and survival. A predator that has lost its sight or with impaired vision can not find food and eventually die. Many predator birds are presented to veterinary clinics due to gunshot injuries, electrical accidents, and trauma [3]. The treatment protocols do not differ much from other domestic mammals, especially to treat ophthalmic diseases as long as anatomo-physiological characteristics of individual bird species are well known. Ocular anatomy may differ between species [2-4]. This variation is required to be known well for a definitive diagnosis and an effective treatment.

Eagle owls *(Bubo bubo)* belong to the owl family of the Raptors family, live in woodlands, steep slopes, cliffs, and mountains, and hunt at night. It is among the endangered species in our as well as some other countries [5,6].

Therefore, necessary measures should be taken in our country for the protection of this species. The main food of the eagle birds is eels, lizards, frogs, and wild birds, some tiny mammals and they hunt mostly at dawn and dusk. Being night hunters emphasizes the importance of the eye in eagle birds [1]. The eye plays a very important role in communication between animals and their environment. All birds have basically similar eyes, but there are several differences that reflect their environmental needs [7]. Knowledge of the ocular anatomy and normal reference physiologic data of the Eurasian Eagle Owl is valuable for the protection of this species and using these birds as an animal model for future research and is critical for the accurate diagnosis of ophthalmic diseases. The most important anatomical difference between owl eyes and the eyes of other birds is that the bulbous is tightly attached to the orbital bone. In addition, it is well established that large interspecies variations exist regarding Schirmer tear test (STT), intraocular pressure (IOP), and central corneal thickness (CCT) values as well as normal microbial flora, especially when dealing with exotic or wild animals. It is also scientifically important that these flora differences are well known [7].

Most lesions in patients who are brought to clinics with complaints of eye or vision problems can be diagnosed by direct and indirect eye examinations, but advanced examination methods are required in some cases. Anatomic and histological structures of the eyes of many animal species such as caretta, cats, dogs, sheep, bears, and camels [8-10] including different bird species [11-13] are well-investigated. Studies on reference values of parameters such as eye topography, corneal thickness, intraocular pressure values, pupil diameter, and lens power belonging to different species such as cats, dogs, and horses have been reported [14]. Studies on ophthalmic ultrasonographic biometry of several avian species and ocular biometry on bald eagle species have been found in the literature [4,8,15,16]. However, the ocular parameters of eagle owls are not clear enough. Based on this, this study aimed to determine the anterior and posterior ocular segment reference values of a total of 12 eyes of 6 eagle owls.

Material and Methods

Ethical Approval

Before starting the study, an application was made to the Animal Experiments Local Ethics Committee of Kafkas University for the necessary permissions. Conditional approval received (Approval No: KAÜ-HADYEK/2020-126).

The study started after the Research Permit obtained from the Nature Conservation and National Parks Directorate of the Republic of Türkiye, Ministry of Agriculture and Forestry in line with the recommendations of the ethics committee (Document No: E-21264211-288.04-3161697).

Case Selection

The animal material consisted of 6 eagle owls, which were brought to Kafkas University Wildlife Conservation and Rehabilitation Center and Kafkas University Veterinary Faculty Animal Hospital for different reasons and did not have any eye problems. Birds with a history of general trauma, prolonged malnutrition, poor general condition, and agony were not included in the study. In 4 birds (3 males, 1 female) used in the study had uninfected open wing wounds and 2 birds (1 male, 1 female) had closed fractures (radius). The sex of the birds was estimated based on their body size. Again, considering their wing structures and body sizes, it was decided that the birds were of adult age.

Ophthalmological Examination

A total of 12 healthy eyes of these birds, 6 right and 6 left, were evaluated. For examinations, benefited from infrastructure facilities of Kafkas University, Veterinary Faculty, Animal Hospital. For the measurement of tear secretion Schirmer II test was used. After one drop of propacaine HCl (Alkaline 0.5% ophthalmic solution, Alcon™, Switzerland) was dropped into the eyes, Schirmer Tear Test Strips (Tear Touch™) were placed in the fornix on the outer 1/3 of both eyes and the results were recorded. To measure the tear break-up time (TBUT), 1 drop of fluorescein dye was dropped into the lower fornix, and the dye was dispersed by closing the eyelids by hand. Then, the disintegration time of the precorneal

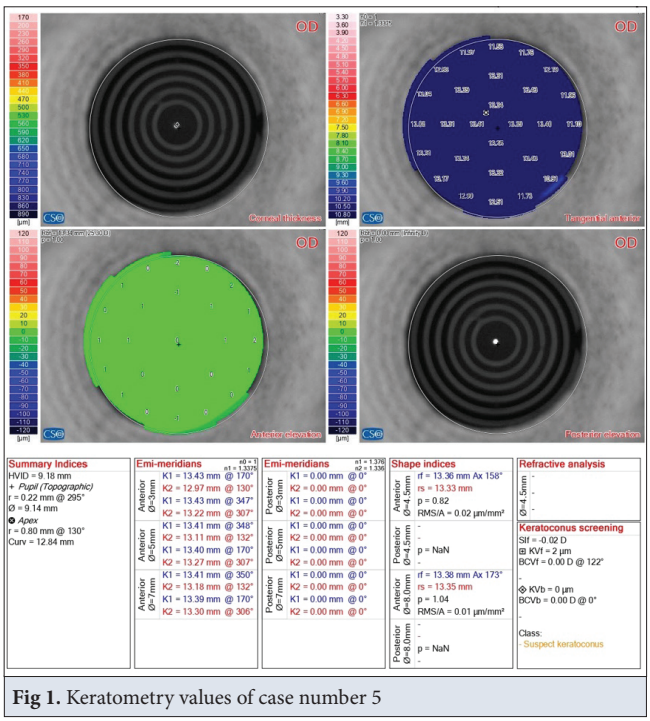

Fig 1. Keratometry values of case number 5

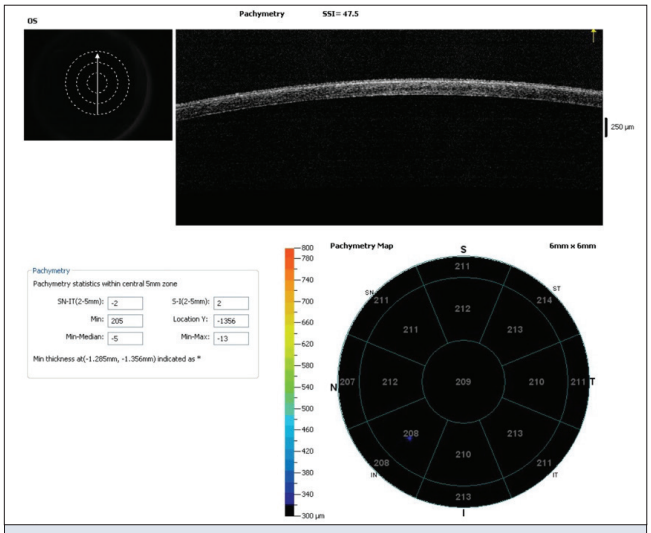

Fig 2. OCT measurement of corneal thickness of case number 2

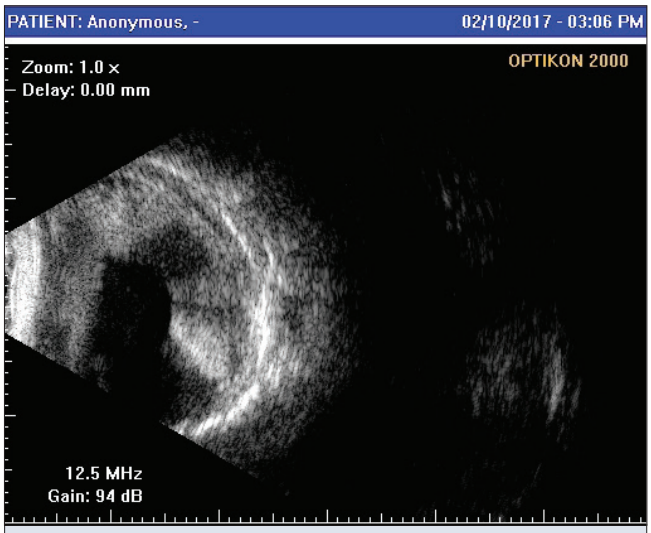

Fig 3. Ultrasonographic appearance of the anterior and posterior segment of case number 4

fluorescein dye in the slit lamp by opening the eyelids, in seconds, keratometric values (K); flattest meridian (K1), and steepest meridian (K2) values in diopter units with manual keratometry, axial lengths in mm with an optical biometric device, lens power in terms of biometrics and diopter units based on manual keratometric values, horizontal and vertical diameters of the corneas were measured with a goniometer in mm, and the results were recorded *(Fig. 1)*. Sirrius 3 dimension rotational Scheimpflug camera topography system (Costruzione Strumenti Oftalmici, Florence, Italy) was used for corneal topography. This technique permits measurement of the cornea in a normal physiologic state (nonanesthetized). Anterior chamber deep (ACD), horizontal visible iris diameter (HVID), pupil diameter (PD), and basic curve (BC) were measured with topography.

The optic disc, tapetal, non-tapetal region, and retina were evaluated with fundoscopic examination and color fundus photographs. The corneal thickness of the eyes was measured with optical coherence tomography (OCT) (RTVue™) *(Fig. 2)*, and intraocular pressure values were measured with an applanation tonometry mounted on a biomicroscope (Topcon CT-800A™). Anterior and posterior segments were evaluated by orbital ultrasonography (Opticon 2000, 15 MHz, 94 dB gain probe) *(Fig. 3)*.

Statistical Analysis

Statistical analyses were performed in the SPSS 18.0 program. First of all, a normality test was performed on the data. Independent Sample T-test was performed for data showing normal distribution, while Mann Whitney U test was used for data not showing normal distribution.

Results

Autorefractometers of all eagle owls were measured as normal. Intraocular pressure values, horizontal corneal diameters, vertical corneal diameters, and central corneal thickness values are given in *Table 1*. The total means of results were found to be 13.81 mmHg, 13.57 mm, 13.20 mm, and 207.83 µm respectively. When comparing the values of IOP, HCD, VCD, and CCT There was no statistically significant difference between the right and left eyes (P>0.05).

Schirmer II test results, TBUT, lens powers, Keratometric K1 and K2 values, and axial lengths in the right and left eyes are given in *Table 2*. Total means of results were found 16.25 mm, 22.08 sec, 25.16 dpt, 25.16 dpt, and 31.10 dpt

Table 1. *Mean ± SEM values of IOP, HCD, VCD and CCT*

Case	Number of Cases	IOP (mmHg)	HCD (mm)	VCD (mm)	CCT (µm)
Right eye	6	14.06±0.38	13.54±0.13	13.13±0.23	208±0.85
Left eye	6	13.56±0.15	13.60±0.13	13.26±0.18	207.67±0.61
Total mean	12	13.81±0.21	13.57±0.09	13.20± 0.14	207.83±0.50
P value	-	0.374	0.567	0.744	0.053

SEM: *Standard error of mean;* ***IOP:*** *Intraocular pressure;* ***HCD:*** *Horizontal corneal diameter;* ***VCD:*** *Vertical corneal diameter;* ***CCT:*** *Central Corneal Thickness*

Table 2. *Mean ± SEM values of Schirmer II test, TBUT, diopters, and AL*

Case	Number of Cases	Schirmer II Test (mm/5 min)	TBUT (Second)	Autorefractometer (Dioptri)	K1 (Dioptri)	K2 (Dioptri)	AL (mm)
Right eye	6	14.67±0.84	23.67±2.04	-0.25x170^0	25.00±0.18	25.08±0.15	31.20±0.24
Left eye	6	17.83±1.01	20.50±0.88	-0.25x160^0	25.33±0.10	25.25±0.17	31.01±0.27
Total mean	12	16.25±0.78	22.08±1.16	-	25.16±0.11	25.16±0.11	31.10±0.17
P value	-	0.334	0.162	-	0.382	0.687	0.020

SEM: *Standard error of mean;* ***TBUT:*** *Tear break up time;* ***AL:*** *Axial length*

Table 3. *Mean ± SEM values of ACD, HVID, PD and BC*

Case	Number of Cases	ACD (mm)	HVID (mm)	PD (mm)	BC (mm)
Right eye	6	3.24±0.00	9.02±0.00	8.31±0.00	10.98±0.00
Left eye	6	3.18±0.01	9.03±0.00	7.63±0.06	10.88±0.01
Total mean	12	3.21±0.01	9.02±0.00	7.97±0.10	10.93±0.10
P value	-	0.141	0.004	0.004	0.748

SEM: *Standard error of mean;* ***ACD:*** *Anterior chamber deep;* ***HVID:*** *Horizontal visiable iris diameter;* ***PD:*** *Pupil Diameter;* ***BC:*** *Basic curve*

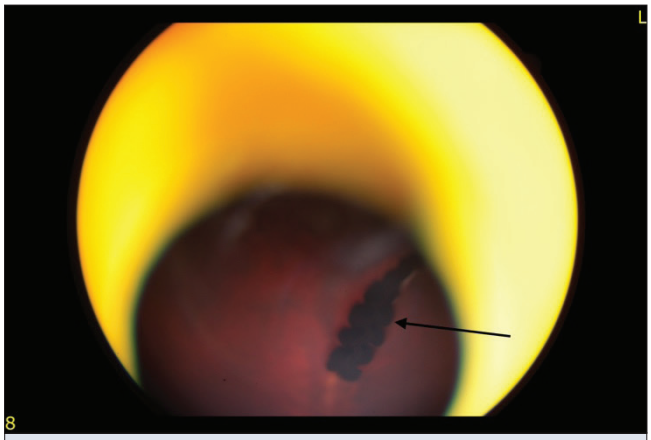

Fig 4. Retinal Imaging: Due to the pecten *(black arrow),* a clear image cannot be obtained in the eyes of the eagle owls (case number 1) during fundoscopic examination

respectively. When comparing the values of the Schirmer II test, TBUT, lens powers, Keratometric K1 and K2 values, there were no statistically significant differences between the right and left eyes (P>0.05) but statistically significant difference were found in axial lengths values (P<0.05).

Anterior chamber depth (ACD), horizontal visible iris diameter (HVID), pupillary diameters, and basic curve values in corneal topography are given in *Table 3*. The total means of results were found 3.21 mm, 9.02 mm, 7.97 mm, and 10.93 mm, respectively. There was no statistically significant difference between ACD and BC values (P>0.05) but HVID and PD values were found to be statistically significantly different (P<0.05).

Lens, vitreous, and pecten were evaluated in anterior and posterior segments in ultrasonography. All structures including retina and choroid were found intact.

In the fundoscopic examination, the retinas of all eagle owls were observed in orange color. Although vascular structures were observed in the deep, very clear images could not be obtained due to the pecten structure *(Fig. 4)*.

Discussion

In this study, in which anterior and posterior segments of 12 eyes of 6 eagles brought to our hospital for different reasons were evaluated, to determine the reference values.

For the study, eagle owls that roam free in nature were not used, only those that were brought to our hospital except for eye or vision problems were used, so the number of cases was limited. Although the number of cases is sufficient to determine the statistical average values, it should be assumed that the reference intervals may change with the evaluations to be made on a larger number of eagle owls.

The mean intraocular pressure has been reported as 13-15 mmHg in humans [17], and 12.3±4 mmHg in cats [18], 10-25 mmHg in dogs, and intraocular pressure values below 20-21 mmHg are also considered normal. In a study by Reuter et al.[19] investigating the intraocular pressure of different species, pressure values was reported as (mmHg±SD) white-tailed sea eagle *(Haliaeetus albicilla),* 26.9±5.8; red kite *(Milvus milvus),* 13.0±5.5; northern goshawk *(Accipiter gentilis),* 18.3±3.8; Eurasian sparrowhawk *(Accipiter nisus)*, 15.5±2.5; common

buzzard *(Buteo buteo)*, 26.9±7.0; common kestrel *(Falco tinnunculus)*, 9.8±2.5; peregrine falcon, *(Falco peregrinus)*, 12.7±5.8; tawny owl *(Strix aluco)*, 9.4±4.1; long-eared owl *(Asio otus)*, 7.8±3.2; and barn owl *(Tyto alba)*, 10.8±3.8 *(Anne reuter)*. IOP values obtained from the study are also similar when compared to IOP in humans, cats, Eurasian sparrowhawks, peregrine falcon, and barn owls but it has been reported that IOP values vary according to age, breed, and certain times of the day [17-19].

Eye shape has been defined as the ratio of corneal diameter (CD) to axial length (AL), and vertebrate eyes have adapted to their environment [3]. Therefore, eye shapes differ in terrestrial vertebrates, reptiles, and birds. Because eagle owls are nocturnal hunters, they have a high CD:AL ratio, meaning they have larger CD and shorter AL [3]. In a study [3] in which the eye axial lengths of different owl species (9 species) were measured, it was reported that the axial lengths ranged from 16.8±07 *(Northern saw-whet owl)* mm to 36.5 mm *(Snowy owl)* according to the species. Eye axial lengths of eagle owls, were measured between 30.9 mm and 31.35 mm. In this respect, the eye axial lengths of eagle owls are similar to the Snowy owl species.

It has been reported that the AL of the eye in humans grows rapidly in the postnatal 18 months and reaches adult size in the next 10 years [20]. Therefore, it has been reported that this distance between the cornea and the retina may vary depending on age. Each of the eagle owls included in our study was in the adult period, and the fact that the AL of the eyes were close to each other supported that each of them was an adult. Since the eagle owls used in this study are adults, data on the AL of the eyes of the young and developmental eagles could not be obtained. In humans, it has been reported that the AL is shorter in anisohypermetropic eyes than in normal eyes, and longer in anisohypermetropic eyes [21]. Determining the mean values of eye axial lengths of healthy eagle owls serves as a reference for the diagnosis of anisohypermetropic and anisomyopic eyes. In this study, it is certain that the diagnosis of the diseases of patients with amblyopia, anisometropia, many eye and vision problems, especially amblyopia, anisometropia, can be made by revealing the reference values.

Recently, anterior segment optical coherence tomography (AS-OCT) has emerged as a new non-invasive and non-contact imaging technique for the anterior segment. AS-OCT provides fast and easy quantitative analysis of various structures by generating high-resolution images using long wavelength (1.310 nm) light [19-23]. A limitation of AS-OCT is that it has incomplete penetration through the pigmented epithelium of the iris, thus making it difficult to obtain accurate images of the ciliary body, lens, and zonules behind the pigmented iris. In this study, anterior chamber depth and angle widths were determined in normal eyes using AS-OCT. Quantitative data were obtained from normal eyes measured by AS-OCT and standardized parameters of anterior chamber configuration were determined. Since OCT is a non-invasive and non-contact method, stress that may occur during examination in eagles is also prevented.

Owl species have a pecten structure in the choroid part of the eye, where blood vessels provide a comb-like appearance. The pecten lies just above the optic nerve head and extends from the retina to the vitreous body. Pecten, also called pecten oculi, has a pigmented structure. In birds, the retinal blood vessels, which supply blood to the eye, have more than undertaken this task. Pecten also helps in the formation of the blood-retina barrier in the eye. There are three different types of pecten in birds. These; according to their morphology, are conical, vaned, and pleated types. The conical type is only reported in the brown kiwi (Apteryx mantelli); the vaned type is present in ostriches *(Struthio camelus)* and rheas *(Rhea americana);* and the pleated form is widely reported in most birds (neognathae) such as quail, black kite, and galah.

The size of pecten in birds varies with the bird's visual needs. Day-active bird species have relatively large and highly complex pecten oculi with many folds, while nocturnal-active bird species have relatively small and simple pecten oculi. The most common type seen in birds is the pleated type [16]. Due to its pectin-pigmented structure, it prevents obtaining a clear image in fundoscopic examination. In our study, a clear image could not be obtained in the fundoscopic examination due to the pecten structure.

In this study, 12 healthy eyes of 6 eagle owls were included, which is the minimum number required for statistical analyses. Before starting the study measurements, a direct eye examination was performed for each eye. In addition, the eye was examined by fluorescein staining to determine if there was a lesion in the cornea. Birds with lacrimation, burrs, generalized infection, prolonged non-feeding, and suffering were excluded from the study. No sedation or anesthesia was used to measure the anterior and posterior segment parameters of the eye.

As a result, it was thought that the results obtained from this study, which was planned to determine the anterior and posterior segment parameters of healthy eagle eyes without vision problems, would be a guide in the differentiation of healthy and pathological eyes and would be a reference for future scientific studies.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (C. Ş. Ermutlu) upon reasonable request.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author Contributions

CŞE, LB, and İÖ conceived and supervised the study. EK, ÖA, BB, HGB, UA, UY, and DGE collected and analyzed data. CŞE, LB, and BB, UY performed the examinations. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Evaluation of Cold Carcasses of Kıvırcık and Romanov Lambs by Geometric Morphometric Method

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Abstract

Carcass discrimination is a very important issue in the evaluation of different sheep breeds as lamb meat for the red meat sector and the study aims to determine the carcass differences of Romanov and Kıvırcık lambs up to 6 months old by 2D geometric analysis. In this study was carried out with six months old 13 Kıvırcık and 16 Romanov lamb cold carcasses. Principal components (PC) that describe the most variation in shape were determined for all samples. A discriminant analysis was performed for the mean shapes of two different breeds. In terms of size (centroid size), Romanov's results were higher. The difference in shape (procrustes distance) was statistically significant for the whole carcass. The shape variations were pretty close for the top view of the whole carcass. It was observed that this analysis was not effective in the differentiation of breeds. The most significant analysis between the two feedings was in the side view of the whole carcass. A total of 26 PCs were obtained from the side view of the whole carcass, 25 PCs from the inside view of half carcass, and 12 PCs from the shape analysis made from the top view of the whole carcass as a result of PCA analysis. PC1 was found to describe for more than 40% shape variation for each view. The lower border of Romanov's rib cage was more ventral according to the results. Also, Kıvırcık had a straighter thoracal and lumbar vertebrae arrangement, while Romanov's was curved. Geometric morphometry can be a useful method for carcass separation.

Keywords: Geometric morphometrics, Principal component analysis, Sheep, Shape analysis, Taxonomy

Introduction

Sheep-breeding in Turkey is carried out by natural methods, and it is traditionally run by small family-type businesses that have great importance in terms of nutrition, income and culture of the rural population [1]. One of the primary incomes in sheep husbandry in Türkiye is lamb as reported and also a preferred protein source for consumers [2]. For this reason, there are many breeds of sheep such as Kıvırcık, Dağlıç, Merino, and Avesi are produced in Türkiye. In addition to these breeds, there are also various imported sheep breeds such as Romanov sheep, Dorper and Suffolk [3]. Among these breeds, some have very important roles in the red meat sector such as Romanov breed, which was raised with the ability to produce multiple offspring, and the Kıvırcık breed, which was raised in terms of meat quality [4]. In Türkiye, it is reported that some of the studies on meat production are carried out in the field of sheep breeding [5].

Unlike traditional morphometric analysis, geometric morphometric (GM) analysis is used to detect shape differences. Shape differences within and between groups can be revealed by statistical methods via GM analysis.

Additionally, GM analysis methods do not deal with size or dimension. GM analysis concentrates on the concept of shape. Anatomical points, curves and contours are used as data sources in GM [6]. Two or three dimension visual data sources which are obtained above mentioned regions can be used for the analyses. The points required for shape analysis from these regions so called landmarks can be 3 types [7]. Type 1 landmarks are landmarks used in anatomical regions that are easy to define. Type 2 landmarks are those used in the most recessed or protruding part of the shape. Type 3 landmarks, on the other hand, are landmarks placed along the curve, also called semi-landmarks.

There are publications stating that breeds and sex discrimination on biological samples have been successfully made and positive results have been obtained in shape analysis with the GM analysis method in recent years [8-12]. It can be revealed whether the shapes are statistically different from each other by GM analysis. Although breeds differences can be demonstrated morphometrically in carcass separation in slaughterhouses, meat warehouses and integrated facilities, this distinction is quite difficult in terms of between breeds differences. Especially the differences between breeds can be reduced to a level that cannot be observed with the naked eye with some corrections made on the carcass. These practices, which allow for unfair profit in the commercial sense, can also cause many unwarranted reservations about meat quality. Shaving body fat, reducing tail fat, reducing shell fat are examples can be given for such negative practices. It is important to overcome this problem with an objective systematic evaluation. For this purpose, it has been tried to understand whether breeds discrimination can be made by GM analysis method on cold carcasses of Kıvırcık and Romanov lambs. It is stated that various researchers conducted studies on cold carcass yields in lambs as our study carried on [5]. Carcass discrimination is a very important issue in the evaluation of different sheep breeds as lamb meat for the red meat sector and the study aims to determine the carcass differences of Romanov and Kıvırcık lambs up to 6 months old by 2D geometric analysis.

Material and Methods

Ethical Statement

The study did not require ethical approval.

Animals

The study was carried out with six months old 13 Kıvırcık and 16 Romanov lamb cold carcasses, obtained from Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa. After slaughter, the carcasses were kept in +4 cooler for 24 h. Photographs of the rested carcasses were taken from 3 different angles. Inspection of carcasses during slaughter was carried out by the veterinarian. There was no pathological finding on the carcasses.

Geometric Morphometry

Images of the carcass from 3 different angles were used for geometric morphometry;

1- Side view of the whole carcass *(Fig. 1)*

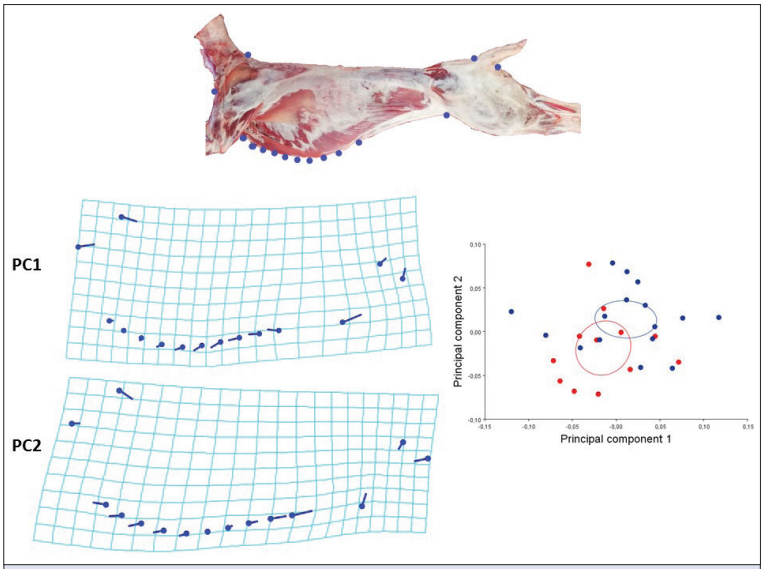

Fig 1. The landmarks used for the side view of the whole carcass (top left), transformation grids for PC1 and PC2 (below left), the distribution of samples according to PC1 and PC2 (right). The points represent the mean shape and the extensions on the dots represent the positive change of the PC value in transformation grids. (Red: Kıvırcık sheep; Blue: Romanov sheep, confidence ellipses for means)

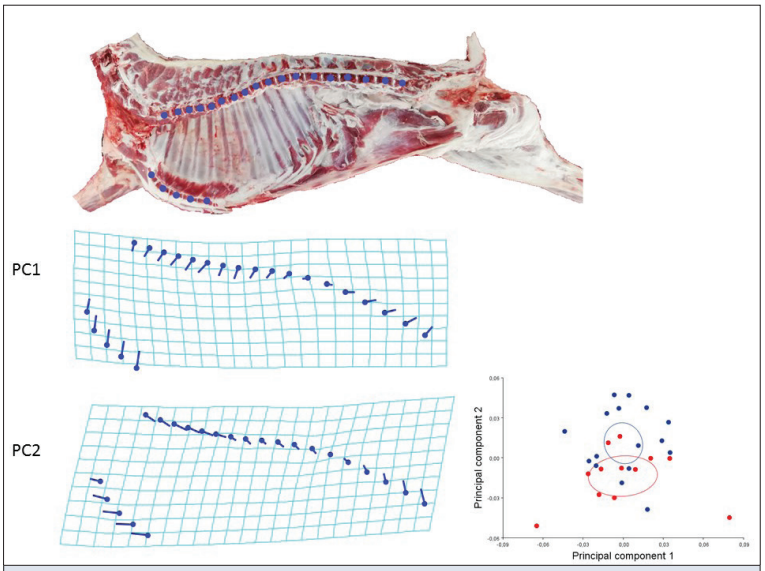

Fig 2. The landmarks used for the inside view of the half carcass (top left), transformation grids for PC1 and PC2 (below left), the distribution of samples according to PC1 and PC2 (right). The points represent the mean shape and the extensions on the dots represent the positive change of the PC value in transformation grids. (Red: Kıvırcık sheep; Blue: Romanov sheep, confidence ellipses for means)

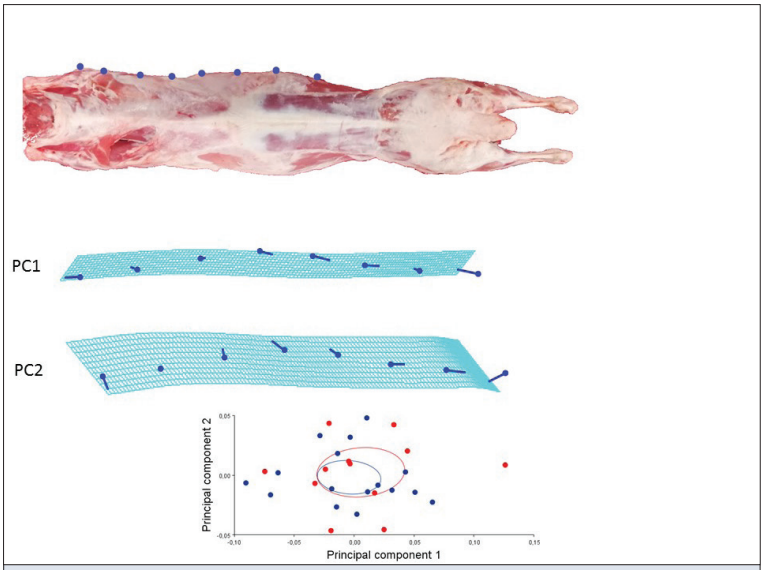

Fig 3. The landmarks used for the top view of the whole carcass (top left), transformation grids for PC1 and PC2 (below left), the distribution of samples according to PC1 and PC2 (right). The points represent the mean shape and the extensions on the dots represent the positive change of the PC value in transformation grids. (Red: Kıvırcık sheep; Blue: Romanov sheep, confidence ellipses for means)

2- Inside view of half carcass *(Fig. 2)*

3- Top view of the whole carcass *(Fig. 3)*

Images were taken by the same person at the same distance (1 meter) for the homogeneity of the analysis. Images were taken via Canon 500D camera and transferred to the computer environment. Photos taken in "jpeg" format were first converted to "tps" format using tpsUtil (version 1.76) [13]. Afterwards, landmarks were used on the shapes using tpsDig2 [14]. The landmarks used in the study are given in *Fig. 1*, *Fig. 2*, and *Fig. 3*. First, points were placed from the initial level of the arm muscles to the lower and upper parts of the neck for the side view of the whole carcass *(Fig. 1)*. Landmarks (Type 1) were placed on the border of the hindlimb with the trunk and the initial upper and lower border of the tail for the rear part. Ten semi-landmarks (Type 2) were used on the lower border of the thorax. Semi-landmarks were used on the center of the trunks of the 13 thoracic vertebrae, the first 5 lumbar vertebrae, and 5 sternums for the inside view of half

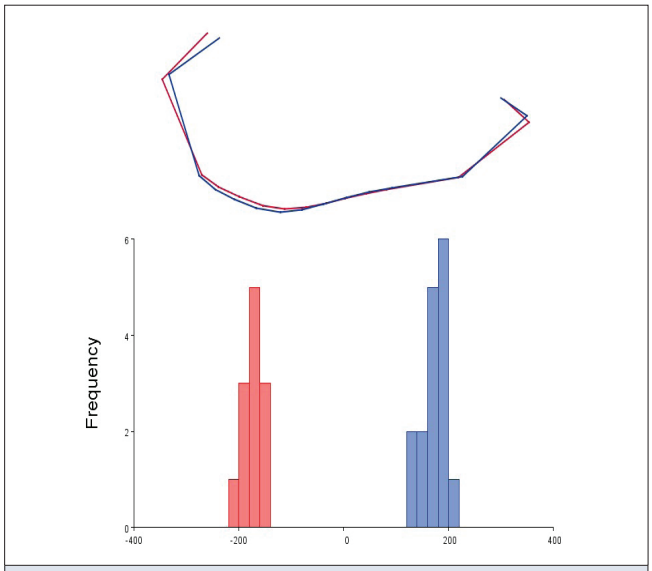

Fig 4. Average shapes for Kıvırcık sheep and Romanov sheep for whole carcass view (above), distinctive scores and frequency for Romanov sheep and Kıvırcık sheep (below). (Red: Kıvırcık sheep; Blue: Romanov sheep)

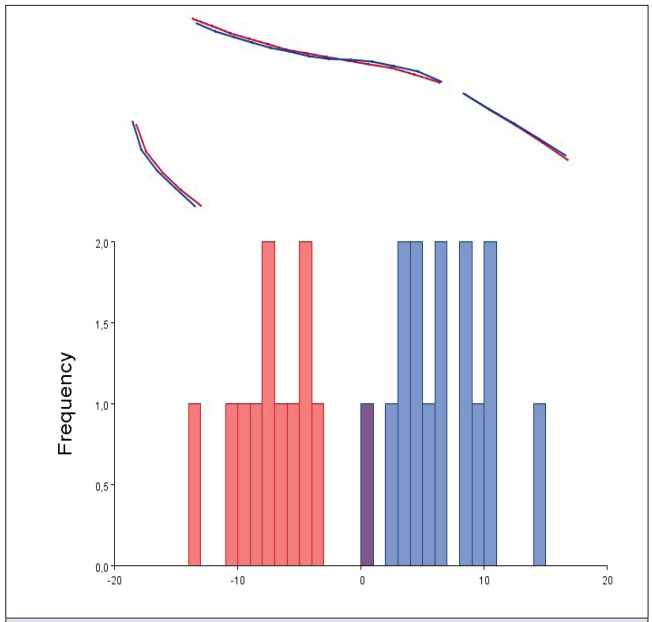

Fig 5. Average shapes of Kıvırcık and Romanov ewes (top), distinctive scores and density of Romanov and Kıvırcık ewes (bottom) for side view of half carcass

carcass *(Fig. 2)*. Finally, 8 semi-landmarks were used to examine the lateral borders of the thoracic cavity in terms of shape for the top view of the whole carcass *(Fig. 3)*.

Statistical Analysis

For the statistical analysis of the study, the morphoJ statistical program was used. First, procrustes analysis was performed to eliminate position and size differences. Then, the average shape and shape variations for all samples used in the study were revealed by principal component analysis (PCA). Principal components (PC) that describes the most variation in shape were determined for all samples. A discriminant analysis was performed for the mean shapes of two different breeds. It was examined whether Kıvırcık and Romanov sheep were distinguished from each other in terms of shape with the discriminant analysis. Procrustes distance and centroid size averages and p values of the breeds were obtained.

Results

A total of 26 PCs were obtained from the side view of the whole carcass, 25 PCs from the inside view of half carcass, and 12 PCs from the shape analysis made from the top view of the whole carcass as a result of PCA analysis. PC1 was found to describe for more than 40% shape variation for each view. The highest PC1 value belonged to the top view of the whole carcass.

PCA analysis results for the side view of the whole carcass are given in *Fig. 1*. The points used for the transformation grids for PC1 and PC2 in *Fig. 1* belong to the mean figure. The extensions of the dots represent how much and in which direction the shape variations of the positive value PCs are. The positive change for PC1 represented a neck structure closer to the chest. The points on the tail and hindlimbs were more distant in shape than the chest. The most important change was in the tail and hindlimb region in terms of PC2. The origin of the tail was thinner in shape and the hindlimb was closer to the origin in increasing PC2. In addition, the increased PC2 value had a wider lower rib cage border in shape. It was seen that Romanov lambs had wider PC1 variation in the distribution of samples according to PC1 and PC2. Conversely, the Kıvırcık lamb had wider shape variation for PC2. Although the difference was not much for PC1, Romanov's mean values for PC2 were higher than for Kıvırcık lamb.

PCA analysis results for analysis of half carcass are given in *Figure 2*. The points used for the transformation grids for PC1 and PC2 in *Fig. 2* belong to the mean figure. The extensions of the dots represent how much and in which direction the shape variations of the positive value PCs are. The positive change for PC1 represented a narrower rib cage. In addition, the lumbar vertebrae were more dorsal with increasing PC1 value. Shape variations were close to each other between Romanov and Kıvırcık sheep in PC1. The increased value for PC2 represented a more anterior sternum line in shape. In addition, the lumbar vertebrae were more dorsal in increasing PC2. Romanov sheep had a high PC2 value, while Kıvırcık sheep had lower.

The PCA results for the top view of the whole carcass are given in *Fig. 3*. The points used for the transformation grids for PC1 and PC2 in *Fig. 3* belong to the mean figure. The extensions of the dots represent how much and in

Table 1. *PCA analysis results. Top 3 PCs describing the highest variation*

Principal Components	Side View of the Whole Carcass (Fig. 1)		Inside View of Half Carcass (Fig. 2)		Top View of the Whole Carcass (Fig. 3)	
	Eigenvalues	% Variance	Eigenvalues	% Variance	Eigenvalues	% Variance
PC1	0.00274632	41.755	0.00084016	40.042	0.00216748	46.805
PC2	0.00146299	22.243	0.00052845	25.186	0.00065114	14.061
PC3	0.00084454	12.840	0.00023674	11.283	0.00059618	12.874
Total PC	26 PC		25 PC		12 PC	

PCA: Principal Component Analysis; PC: Principal Components

Table 2. *Results of CS and shape*

View	Effect	SS	MS	df	F	P
Side view of the whole carcass	CS	53407.501005	53407.501005	1	0.16	0.6898
	Shape	0.01547913	0.0005953510	26	2.35	0.0002
Inside view of half carcass	CS	5661.371481	5661.371481	1	0.03	0.8719
	Shape	0.01259594	0.0002999033	42	1.08	0.3374
Top view of the whole carcass	CS	201676.707297	201676.707297	1	4.57	0.0421
	Shape	0.00197158	0.0001642985	12	0.43	0.9528

CS: Centroid Size; SS: Sum of Squares; MS: Mean Squares; df: Degrees of Freedom

Table 3. *The distribution of the samples as a result of the discriminant analysis*

Samples	Side View of the Whole Carcass (Fig. 1)		Inside View of Half Carcass (Fig. 2)		Top View of the Whole Carcass *(Fig. 3)*	
	Kıvırcık	Romanov	Kıvırcık	Romanov	Kıvırcık	Romanov
Kıvırcık	12	0	11	1	12	0
Romanov	0	16	0	16	3	13

which direction the shape variations of the positive value PCs are. For the top view, the variation changes for the Romanov and Kıvırcık sheep were very close to each other. Top view analysis of the whole carcass was less successful in distinguishing the two breeds than analyses for other views of the carcass.

Centroid size (CS) and shape averages and standard deviations are given in *Table 2*. In terms of size (CS), Romanov's results were higher. This difference was statistically different for top view, but statistically insignificant for other analyses. The difference in shape (procrustes distance) was statistically significant for the whole carcass. For other analyses, the difference was statistically insignificant.

The distribution of the samples as a result of the discriminant analysis is given in *Table 3*. According to these results, the two breeds were completely separated from each other with the side view of the full carcass. In the analysis of half carcass, 1 Kıvırcık showed Romanov carcass shape features. In the analysis of the carcass images from the top, the distribution was correct for the Kıvırcık sheep carcass samples, but 3 Romanov sheep samples showed shape characteristics of Kıvırcık sheep carcass.

The average shapes of Kıvırcık sheep and Romanov sheep carcass for the side view of the whole carcass are given in *Fig. 4*. According to these results, the forelimb of the Kıvırcık sheep was more anterior and lower than the Romanov sheep. Also, the tail border of the Kıvırcık sheep was observed lower and backwards. The border below the chest was lower in shape in Romanov sheep.

The average shapes of Kıvırcık and Romanov sheep for the inside view of the half carcass are given in *Fig. 5*. According to the results, the lower border of the rib cage of the Romanov sheep was still in the abdomen. Also, the Curly sheep had a flatter thoracic and lumbar arrangement, while the Romanov sheep had a curved arrangement.

Discussion

Various studies have reported that carcasses of animals raised with different feeds and diets change many factors such as carcass, meat quality, meat color, and meat flavor [15-17]. It has been reported that information on other yield characteristics such as carcass yield and meat quality characteristics of the Romanov sheep breed should be completed [18], and in this study has brought a different perspective to the subject and revealed the shape

differences between Kıvırcık and Romanov cold carcasses comparatively. Priolo et al.[19] reported the evaluation of carcass structures in animals fed with different feeds and feeding techniques by means of a visual evaluation method. It was also observed that the width of the carcass was wider in the barn-fed ones. Ekiz et al.[20] reported that carcass quality is an important factor in breeds. When the shape differences were examined in our study, it was seen that there were differences between the studied breeds when the PC1 and PC2 analyzes were examined depending on the breeds and different variables. Cameron found that there was a high positive correlation between arm dissection and the amount of meat, fat, and bone in the whole carcass [21]. In another study, it was reported that leg and arm dissections gave effective results in determining the meat, fat, and bone ratios of the carcass [22]. Wilson et al.[23] reported that there is a positive correlation between carcass size and large body limb bones in ruminants. Demir reported a high correlation between the thigh for total meat estimate and the waist region for total fat in Kıvırcık sheep [5]. Manuta et al.[24] reported that cows, sheep, and horses have higher PC1 values compared to horses in their studies on olecranon, and the tuberosity of olecranon is wider. In this study, the view of the carcass shape from different angles was studied. The increased PC1 value in the whole carcass view gives the ventral view of the thorax. Half carcass, Ascending PC1 gives a narrow appearance. The increase in the lumbar vertebrae affects the carcass width and causes changes in PC1 and PC2 values. In studies, it was seen that the positive growth effect of the bone on the carcass was also observed in our studies. Akçapınar [25] determined that the correlations of thigh and arm meat amount of Kıvırcık sheep showed a high positive correlation with whole body meat amount. In this study, it was observed that the effect of the amount of leg and arm meat on the whole carcass changed according to PC1 and PC2 analyses.

While geometric morphometry reveals the differences between the races, it also reveals the structural and anatomical differences between the breeds. Manuta et al.[26] reported that the increase in PC1 and PC2 values varies in different anatomical structures. For example, PC1 increase indicates narrow acetabulum whereas PC2 increase indicates margin of acetabulum. In the study of Hadžiomerovic et al.[10] with ear ossicles, increased PC1 was seen in the caput mallei of malleus, while PC2 increased in the caput mallei of malleus. Szara et al.[27] know that in his study on Japanese Quails, PC1, PC2 and PC3 increase and decrease in the same and different anatomical structures reveal differences. In this study, the PC1 and PC2 increase, for example, the change in the sternum of the carcass, was found to be equivalent to other studies in the study conducted with 3 different appearances of the carcasses. It is known that the statistical difference between breeds and sex in the studies performed on Centroid size reveals the closeness of the central figure to the central figure, which increases the quality of the studies. Parés-Casanova and Xènia reported that in comparison of the sphenoid bone of sheep and goats, sheep were larger in shape, and the statistical difference in centroid size and shape was quite significant ($P<0.001$) [28]. When we compared Romanov and curly carcasses in this study, it was revealed that the Romanov was larger in shape. Manuta et al.[26] reported on crossbred cats, stated that the difference between the pelvis and the female male was statistically insignificant ($P>0.05$). Manuta et al.[24] found that Shape and centroid size were statistically significant in horses, sheep and cows in their study on Calcaneus ($P<0.001$). Gündemir et al. reported that there was no statistical difference in dorsal and lateral view centroid size in their study among cat breeds, but the dorsal and lateral statistical difference between cat breeds in shape was quite significant ($P<0.001$) [29]. Ojanguren-Affilastro et al.[30] found that Centroid size and shape were statistically significant in their study on scorpions. In this study, while the whole carcass centroid size of Romanov and Kıvırcık sheep is statistically significant ($P<0.05$), it is highly significant in terms of shape ($P<0.001$). The centroid size of the top view was statistically significant ($P<0.05$). If there is a statistical difference in centroid size and shape between breeds in the studies conducted, the data we have obtained shows that there is a statistical difference like other studies.

Gürbüz and Demiraslan [31], in their study on the incus of horses and donkeys, looked at canonical variance analysis and revealed the differences in anatomical structures between the two breeds. They reported that the corpus inducus was flatter and the top of the crus longum was wider in donkeys. Manuta et al.[26] reported that the line terminalis is wider in females in their study in the cat pelvis. He also determined that the linea terminalis was more prominent on its dorsal side as a specific difference. Casanova and Miquel reported that gender discrimination can be made in geometric morphometric examinations of the dorsal skulls of White Rasquera goats and that the sagittal points of the viscerocranium provide the greatest contribution to this distinction [32]. Yaprak et al.[33] The skulls of Hair, Honamlı, Kilis and Saanen goats were examined geometrically morphometrically and it was seen that it was possible to distinguish between goat breeds. The most prominent points of deformation are the caudo-oral corner of the margo alveolaris of the III molar in females and the meatus acusticus externus in males. Dörtbudak et al. reported that two different fish breeds had anatomical differences in their study on otoliths of fish [34]. As in other studies conducted in this study, anatomical differences were observed between breeds. For example, in the whole

carcass, the front part of the Kıvırcık sheep is seen lower and behind compared to the Romanov sheep. Anatomical differences were also seen in the data obtained in top view and half carcass.

In conclusion, this study is the first new in this field. It aimed to add a new breath with the contributions of the departments of anatomy and food by presenting the multidisciplinary contributions of different scientific fields. It was examined that it was not possible to make a separation between the two breeds by using the photographic method. The shape variations were pretty close for the top view of the whole carcass. It was observed that this analysis was not effective in the differentiation of breeds. The most significant analysis between the two feedings was in the side view of the whole carcass. This difference was also statistically significant in the ANOVA results. After the shape variations, a discriminant function was performed for the two breeds. In these results, the side view of the whole carcass image was distinctive for the two feedings. In the analysis made for the top view within the discriminant function, the error was high in classification. Geometric morphometry can be a useful method for carcass separation.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author (O. P. Choudhary).

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Ethical Statement

The study did not require ethical approval.

Competing Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, M.G.G and A.E.Y, methodology, B.C.G; M.G.G and A.D software, M.G.G; B.C.G and A.E.Y.; validation, M.G.G, formal analysis, M.G.G and B.C.G, investigation, S.K.B, resources, K.M; data curation, E.O. and M.G.G, writing original draft preparation, B.C.G and M.G.G; writing-review and editing, M.G.G; B.C.G and O.P.C; visualization, M.G.G and B.C.G; funding acquisition, O.P.C. All authors have read and agreed to the published version of the manuscript.

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Research Article

Allometry and Atlas Shape Analysis Between Tekir and Mix-breed Cats

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Abstract

The first cervical vertebra, the atlas, connects the skull to the body and plays a crucial role in supporting the skull and holds a distinct position within the atlanto-axial complex. In this study was aimed to examine the shape variation of the atlas in cats and to reveal shape changes related to variation in size via geometric morphometry. A total of 61 (31 mix-breed cats, 30 Tekir cats) cats were used. Shape differences were examined both for mix-breed cats and Tekir cats and between sexes. However, it was seen that the shape difference between species (P:0.1644) and between sexes (P:0.4801) was insignificant according to the discriminant function results. The average shape and shape variations of the atlas were obtained for all samples. A total of 52 principal components were obtained. PC1 explained 28.39% of the total variation. Tekir cats showed more shape variation than mix-breed cats for results of PC1 and PC2. Mix-breed cats had a larger *ala atlantis* than Tekir cats as shape. *Incisura alaris* were deeper in shape in mix-breed cats. *Foramen vertebrale laterale* of the male was more medial in shape than female. It was performed a multivariate regression of the procrustes coordinates as shape variables on the log-transformed centroid size values as a size variable to analyze the allometry of atlas for all samples. However, allometry results were statistically insignificant (P:0.3579). In this study, encompassing breed and sex comparisons in cats, will serve as a pioneering effort in future atlas research, offering substantial data concerning cat atlas bones.

Keywords: Geometric morphometry, shape variation, taxonomy, veterinary anatomy

Introduction

The atlas exhibits distinctive anatomical features when compared to the other cervical vertebrae [1] and plays a crucial role in supporting the skull and holds a distinct position within the atlanto-axial complex [2]. Due to its significant role in the region, this complex is one of the critical areas where surgical interventions are performed for congenital anomalies or trauma-related issues. Various surgical techniques are currently employed in human medicine to address instability in the atlanto-axial complex caused by a range of traumatic and nontraumatic conditions. However, during these procedures, there is a risk of causing harm to vital structures [1].

The importance of atlas morphometric data is valuable for enabling the safe execution of the mentioned procedures and the data is crucial for ensuring the secure implementation of the procedures in question [1,2]. There are numerous morphometric studies conducted on the human atlas bone, focusing on topics such as the general structure of the atlas and its anatomical features [2,3], asymmetry [4], as well as relationships among populations and sexes [5]. Some researchers have obtained materials using more modern methods, including 3D scanners, and have conducted analyses using the geometric morphometric approach, which is also becoming popular nowadays [6].

Studies on the morphology of the atlas bone in humans are generally conducted with the purpose of providing information for surgical interventions, and the same holds true for animals as well. In a study, African Giant Rat vertebrae were examined, and it was noted that the spinal canal is widest, particularly in the region where the atlas bone is located [7]. Another study on the axial skeletons of African lions, the axial bones of the animals were morphologically examined, and it was reported that the structures of their atlas bones resemble those of domestic cats [8]. There are also studies that compare atlases macro-anatomically with different species [9]. The

mentioned studies provide valuable insights that can serve as fundamental knowledge for surgical endeavors, and there are also studies, such as the one by Parry et al.[10], which specifically focus on addressing these goals, like the presence of conditions like incomplete ossification in the atlas that could potentially pose significant neurological issues in dogs. Beside bone morphology research on sexual dimorphism using bone morphometrics in animals is a highly regarded and frequently conducted study. There is a study conducted on the differences between sexes using linear measurements taken from Gazelle pelvic computed tomography images [11]. In addition to classical linear measurements, sexual dimorphism studies have also been conducted in red foxes [12], sheep [13], Japanese quails [14], and cats [15,16] using geometric morphometric methods.

Bones find utility across a diverse spectrum of applications, spanning disciplines like anatomy [17], forensic science, anthropology, the study of evolution [16], zooarchaeology and anatomy education [11]. When considering the atlas bone, it is a scientific fact that there can be morphological differences among species, breeds, and sexes. In this study on the atlas bones of mix-breed cats and Tekir cats employed 3D tomography models and geometric morphometrics to reveal both interbreed and sex-based differences. The study aims to address this gap by examining differences between breeds and sexes in cat atlas bones, using modern techniques such as 3D tomography images and geometric morphometrics, in an effort to provide some data to alleviate this deficiency.

Material and Methods

Ethical Approval

Prior to conducting tomography scans, an "Informed Consent Form" was obtained from the animal owners, and the required ethics committee report for the study was obtained from the Istanbul University-Cerrahpasa Veterinary Faculty Ethics Committee (Report Number: 2022/38).

Animals

Sixty-one cats, which were over 1 year old, were used in the study. The average age of the cats was 3.79, and their average weight was 3.21 kg. Orthopedically healthy cats were used in the study, and their examination was performed before computerized tomography.

Modelling

Computed tomography scans were taken using "Siemens Somatom Scope vc30b". Scanning parameters for all samples were 0.6 mm slice thickness, 110 kV, and 28 mA, total scanning time app - 14 sec. After the scanning process was completed, the images were transferred to the computer and the segmentation process was performed. 3D models of atlas were made using the 3D Slicer (5.1.0 version) program. Soft tissues were removed from the image using the software. This way, atlas images of the samples were obtained from live animals, and then the dorsal side of the 3D specimens was photographed.

Landmarks

Images were obtained from all samples from the same angle and saved to the computer in ''pnp" format. 61 images were converted to ''tps" format using tpsUtil (version 1.74). Twenty-eight Landmarks were used in the study. TpsDig2 (version 2.32) was used to insert the landmarks into the images [18]. Nomina Anatomica Veterinaria was used as a base for the anatomical terms of the landmarks used in the study *(Fig. 1)*.

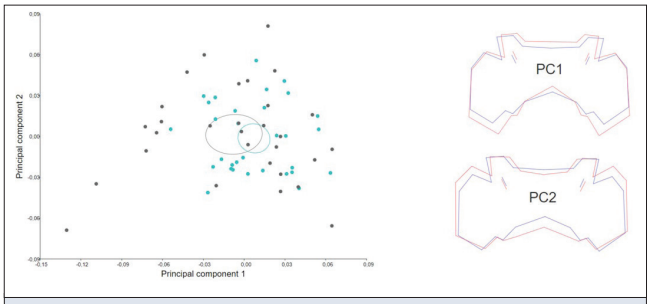

Fig 1. Scatter plot of PC1 (28.39%) and PC2 (16%) of atlas of cats in dorsal view (left) and confidence ellipses for means (Mix-breed cats: Green, Tekir cats: Grey). Wire-frame warp plots of changes in the atlas (right), as mapped by 28 landmarks. Blue outlines represent the mean shape configuration, while the red outlines show the shape changes associated with the positive extremes of the PC axes

Statically Analysis

"Morphoj" software was used for geometric morphometric analysis [19]. Principal component analysis (PCA) was performed to reveal the shape variation of the atlas. The discriminant function (DF) was applied to reveal the differences statistically for cat species and sexes. Incorrect grouping results were obtained by applying cross-validation. Average shapes between groups were examined. The intergroup procrustes distance and mahalanobis distance were obtained. It was performed a multivariate regression of the procrustes coordinates as shape variables (the regression score) on the log-transformed centroid size values (centroid size) as a size variable to analyze the allometry of atlas for all samples. Results are reported as a percentage value of the explained total shape variation from the size variation. Levels of statistical significance were computed by permutation tests, using 10,000 random permutations.

Results

Principal component (PC) analysis was carried out using a total of 61 cats. A total of 52 principal components were obtained. PC1 explained 28.39% of the total variation.

Table 1. Principal components		
Principal Component	**Eigenvalue**	**%Variance**
1	0.00167566	28.39
2	0.00094453	16.00
3	0.00044805	7.591
4	0.00035718	6.051
5	0.00035176	5.959
52	0.00000016	0.003

This value was 16% in PC2 and 7.59% in PC3. Principal components explaining the highest variation are given in *Table 1*.

Scatter plot of PC1 (28.39%) and PC2 (16%) of atlases of cats in dorsal view are given in *Fig. 1*. This scatter plot explained 44.39% of the total variation. Mix-breed cats mean PC1 value was higher than Tekir cats. The mean PC1 value of Tekir cats were negative. When considering the average values for Mix-breed cats and Tekir cats with respect to PC2, they are close to each other, although PC2 is found to be slightly higher. Tekir cats showed more shape variation than mix-breed cats for results of PC1 and PC2.

Fig. 1 shows wire-frame warp plots of changes for PC1 and PC2, with the average shapes in blue. The increased value for PC1 represented the larger *ala atlantis* posteriorly in shape. The borders of the *foramen vertebrale laterale* were more medial with increasing PC1 value. Also, the posterior border of the *arcus vertebralis* was narrower. *Incisura alaris* were also close to medial in shape. The increased value for PC2 represented the larger *ala atlantis* anteriorly in shape. The borders of the *foramen vertebrale laterale* were more lateral with increasing PC2 value. Also, the posterior border of the *arcus vertebralis* was wider. *Incisura alaris* was also close to medial for PC2.

The changes for PC1 and PC3 are shown in wire-frame warp plots *(Fig. 2)*. The shape of *ala atlantis* was smaller and narrower in both anterior and posterior with the increasing value of PC3. The borders of the *foramen vertebrale laterale* were found to be more medial with increasing PC3 value, while the posterior border of the

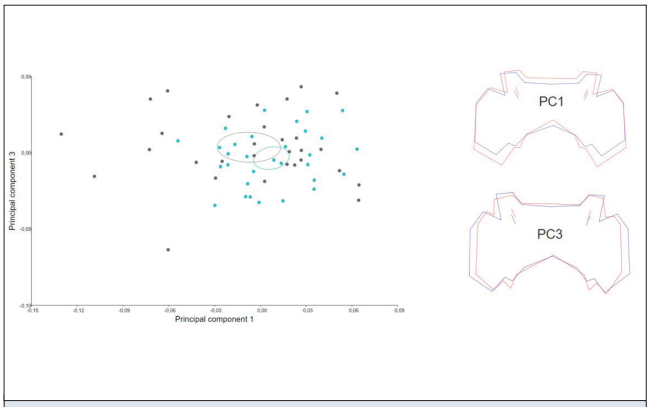

Fig 2. Scatter plot of PC1 (28.39%) and PC3 (7.59%) of atlas of cats in dorsal view (left) and confidence ellipses for means (Mix-breed cats: Green, Tekir cats: Grey). Wire-frame warp plots of changes in the atlas (right), as mapped by 28 landmarks. Blue outlines represent the mean shape configuration, while the red outlines show the shape changes associated with the positive extremes of the PC axes

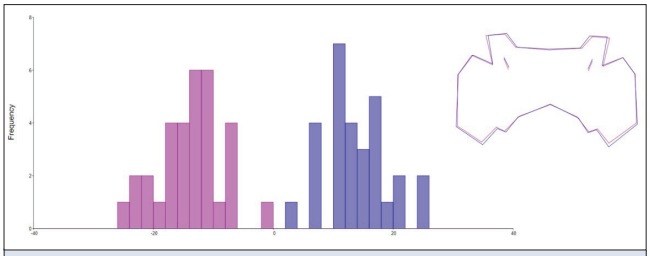

Fig 3. Discriminant function. Wire-frame warp plots of changes for sexes. Female: pink; Male: blue (Procrustes distance: 0.02585845; Mahalanobis distance: 5.2789; T-square: 423.9352; P:0.4801)

arcus vertebralis is closely matched the average width. The position of the *incisura alaris* was closer to the medial aspect compared to the mean shape.

In *Fig. 3*, wire-frame warp plots of changes and average figures for both sexes are given. According to the discriminant function distribution, it was seen that the male and female were separated from each other for the samples used in the study. However, this distinction was statistically insignificant (P:0.4801). Even though the samples were completely separated according to the discriminant function, it was seen that not all of the samples were classified correctly according to the cross-validation results *(Table 2)*. Male cats had a larger *ala atlantis* than females, with an average atlas shape. This enlargement was seen to occur at the caudal part of the

Table 2. Discriminant function and cross-validation prediction accuracy						
Groups		**Discriminant Function**		**Cross-validation**		**Rates (%)**
		Female	**Male**	**Female**	**Male**	**Prediction Rate (%)**
Sex	Female (n:32)	32	0	14	18	43.75
	Male (n:29)	0	29	17	12	41.38
Breed	Mix-breed (n:31)	31	0	21	10	67.74
	Tekir (n:30)	0	30	14	16	53.33

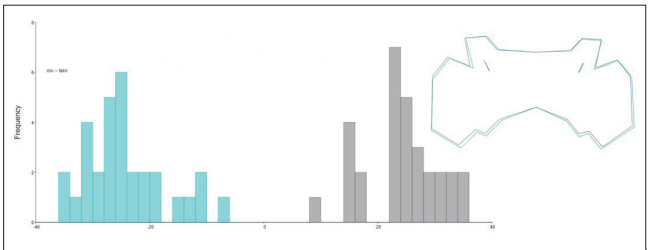

Fig 4. Discriminant function. Wire-frame warp plots of changes for mix-breed cats and Tekir cats. Mix-breed: Green, Tekir: Grey (Procrustes distance: 0.02255164; Mahalanobis distance: 6.9517; T-square: 736.7693; P:0.1644)

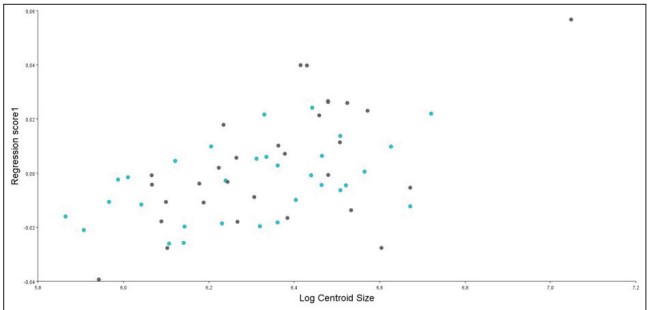

Fig 5. Regression trajectories for both sexes of mix-breed and Tekir cats by relating the centroid size Log-transformed values (Log Centroid Size) vs. the shape scores (the regression score obtained from the Procrustes coordinates). Regression of shape was not significant (n: 61; P:0.3579; 1.74 % of shape variation explained by size variation)

atlas. In addition, the *foramen vertebrale laterale* of the male was more medial in shape than female.

In *Fig. 4*, wire-frame warp plots of changes and average figures for mix-breed cats and Tekir cats are given. According to the discriminant function distribution, it was seen that the mix-breed cats and Tekir cats were separated from each other for the samples used in the study. However, this distinction was statistically insignificant (P:0.1644). Even though the samples were completely separated according to the discriminant function, it was seen that not all of the samples were classified correctly according to the cross-validation results in terms of breed *(Table 2)*. Mix-breed cats had a larger *ala atlantis* than Tekir cats, with an average atlas shape. This enlargement was seen to occur at the back of the atlas. Incisura alaris were deeper in shape in mix-breed cats.

Discriminant function and cross-validation results are given in *Table 2*. According to the discriminant function, samples were completely separated both between the sexes and between the species. Although, according to the cross-validation for both sex and breed discrimination, the results were not satisfactory. Regarding sex discrimination, 14 female cats were categorized correctly, while 18 were categorized as male within the female group. Within the male group, 12 were categorized as male, and 17 were categorized as female.

It was performed a multivariate regression of the procrustes coordinates as shape variables (the regression score) on the log-transformed centroid size values (centroid size) as a size variable to analyze the allometry of atlas for all samples. However, allometry results were statistically insignificant (P:0.3579) *(Fig. 5)*.

Discussion

Our study was conducted on a total of 61 cat atlases using geometric morphometric methods to reveal the shape differences between sexes and species on the atlas bone. When examining the shape differences among breeds in our study's DF results, it was observed that the *ala atlantis* in mix-breeds cats is wider towards the caudal direction compared to Tekir cats. This shape difference is also clearly evident in PC1 shape variation and slightly elevates the mix-breed cats PC average. It was observed that the *incisura alaris* is more medial in DF for mix-breed cats, and this reflects on PC1, PC2, and PC3 shape variations. Furthermore, the DF results also indicate that the posterior border of the *arcus vertebralis* is wider in mix-breed cats compared to Tekir cats. This variation is evident in PC2, which explains 16% of the shape variation.

Results of this study indicate that the atlas bone does not possess sufficient morphological differences statistically to distinguish between sexes. The lack of distinct sex differences in atlas for domestic cats is present in another article that have PCA clustering graph of overall cervical bone shapes showing there are not much discrimination between sexes [16]. Additionally, it has been stated that the average accuracy for sex prediction for the atlas is 63.10%. Although the bones show a complete separation by sex in the DF results in our study, when cross-validation is applied, it is observed that the rates decrease, and an average prediction accuracy of 42.57% is achieved. These results indicate that the shape changes in the atlas are not sufficient for sex predictions. In the same study [16], linear measurements were also taken, and the difference between sexes was found only in the measured caudal articular surface distance, which was statistically significant. Another measurement that is taken from the dorsal part of the bone (as our study contains dorsal view of the bone) was cranial articular surface distance and transverse process length (in cranio-caudal direction) and were statistically insignificant. A study conducted on the vertebral bones of domestic cats by Boonsri et al.[16] concludes that gender has a minimal impact on the cervical vertebrae, and this result is in line with the findings of our study. However, it is mentioned that there is a statistically significant difference between sexes for the caudal articular surface distance, and the shape differences in this study support the caudal expansion of the atlas in males compared to females.

Considering the crucial role of the neck as the cranium's support, the proportionate weight of the cranium in relation to the total body weight remains consistent between males and females in human [20]. In a study encompassing the analysis of human atlas bones, conducted through three-dimensional scanning and utilizing geometric morphometric methods on digitized images, it has been demonstrated that sex differences are statistically insignificant [6]. However, a morphological study conducted on Thai individuals' (human) atlas bones has indicated statistically significant sex differences [5]. In a study conducted by Parry et al.[10], which included various dog breeds and involved 120 dogs, the shape variations of the atlas were examined. Despite the differences among breeds, it was reported that no significant relationship could be found among the shape differences in the atlases. However, in the same study, it was mentioned that the length of the transverse process of the atlas increases with the weight of the dogs. In an anatomical study conducted on elephants [21], the cervical vertebrae of elephants were compared to those of horses and cattle in terms of their structures. It was observed that the cervical vertebrae of elephants are shorter in length compared to other species, and this has been associated with the need for a shorter neck to support their heavy heads. Additionally, it was noted that the width of the atlas bone in elephants is nearly as wide as the animal's neck. The lack of statistical significance in the shape variations in our PCA and DF analyses, despite the shape differences, has led us to consider that this may be due to the fact that the weights of the cats, despite their different breeds, are relatively close to each other. It is a reasonable hypothesis that the ambiguity in sex-related differences associated with head weight in humans and body weight in dogs and elephants could also apply to cats. In addition to head and body weights, among the many breeds of cats with various variations [22-24], it is believed that sex-related differences in atlas shape among cat breeds, similar to humans, may be associated with genetic backgrounds.

This study was based on the shape analyses performed on atlas images obtained from cats and aims to examine whether there are shape differences between sexes and breeds. Discriminant function analysis was conducted to analyze the differences between sexes, and it was observed that the shape differences between sexes resulted in a complete separation of the sex groups. However, during cross-validation analysis, individuals were poorly classified into their respective sex groups. As a result, the obtained P-value from the discriminant function analysis was found to be statistically insignificant (P:0.4801). Similar observations were made for breed differentiation, with a resulting P-value of 0.1644. The analyses revealed that there is no significance in terms of sex and breed differences among atlas bones. Considering the critical importance of the atlas bone's position, there is a need for more data on differences between cat breeds or sexes. In this context, our paper offers substantial data concerning cat atlas bones. We hope that our study will serve as a pioneering effort in future atlas research, encompassing breed and sex comparisons in cats.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (Z. Mutlu) on reasonable request.

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Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Authors Contributions

EÖ and ZM: Conceived and supervised this study; EÖ, BU, YA, and ZM: Collection of the data; EÖ and KA: Statistical analysis. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

A Novel "Onder Speculum" to Visualize and Retract the Cervix During Transcervical Procedures in Small Ruminants

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Abstract

The visualization of the cervix and subsequent manipulations during transcervical applications in sheep might require a degree of straining reflexes while using the Collin Speculum. Therefore, this study aimed to develop and to evaluate the feasibility of a new alternative speculum design for non-surgical assisted reproductive procedures in small ruminants. Collin speculum (CS) and the new "Onder speculum" (OS) were used to assess cervical entrance in 30 ewes. The OS efficiency for a successful cervical retraction was also evaluated. Data showed that the cervical entrance was visible in 8 out of the 30 females with CS, while it was observed in all the animals when using OS ($P<0.001$). The latter was successful in the retraction of the cervical entrance in 10 randomly selected animals out of the total. To conclude, the newly developed speculum effectively supported the implementation of non-surgical assisted reproductive procedures in small ruminants and was also considered to have the potential to facilitate non-invasive reproductive techniques in these species.

Keywords: Small ruminants, Speculum, Cervix entrance

Introduction

Small ruminant breeding has a significant role in some nations with regards to animal-based food intake and holds a crucial place in human nutrition. In addition to the production of meat and milk, it also serves as a great contributor to the country's economy through its involvement in the manufacture of wool, mohair, and leather. Assisted reproductive techniques are frequently employed to regulate the reproductive processes in sheep exhibiting seasonal breeding patterns [1-3]. Artificial insemination (AI), a prominent biotechnology approach within assisted reproductive methods, involves the introduction of fresh, cooled or frozen male gamete cells into female animals [4]. Another assisted reproductive strategy employed to enhance productivity in sheep breeding falls within the realm of embryo production. Sheep that are commonly chosen as donors in embryo production undergo superovulation techniques. After this process, the embryos pass through a series of flushing processes before being transferred to carrier animals [5,6]. Surgical techniques are generally used in the context of AI and embryo production procedures in sheep, mostly owing to the intricate nature of the cervix. Nevertheless, apart from the constraining prerequisites associated with surgical techniques, such as the considerable cost of instruments and the necessity for highly skilled staff, there is also the potential occurrence of adhesions subsequent to surgical treatments, which can lead to numerous adverse outcomes that detrimentally impact the wellbeing of animals. For this reason, many researchers are currently engaged in the exploration of non-invasive procedures and their implementation in ovine assisted reproductive methodologies [7-9].

Reproductive management in small ruminants remains a focal field of interest among scientists, mostly because of the challenges associated with cryopreserving semen

as well as the complexities involved in AI and multiple ovulation and embryo transfer (MOET) procedures [10-12]. In the context of AI and embryo production in cows, practitioners have the ability to manipulate the cervix by contacting it through the rectum using one arm while concurrently managing catheters with the other hand [13]. However, the structure of the cervix has been identified as having a particularly hard aspect for practitioners, namely in some techniques such as AI and MOET procedures which limit their use [8,9,11]. Therefore, in non-surgical embryo production in sheep, researchers describe the initial step as retracting the cervical line to align with the vaginal entrance line, thereby facilitating visibility of the cervical opening. Following this phase, cervical manipulations could be conducted [8,14].

The duck-billed speculum is mostly used in AI techniques conducted on sheep [15,16]. According to different studies, the CS is used in procedures involving embryo flushing and transfer, which necessitate cervical manipulations. In this particular instrument, the speculum can be removed from the vagina subsequent to the stabilization of the cervical entrance, achieved by employing forceps directed through the vaginal canal [8,17,18]. According to reports, it is expected that the insertion of the speculum into the vaginal cavity will cause pushing reflexes in the animal. As a result, the cervical opening is predicted to shift slightly towards the dorsum to allow visualization of the cervical entrance [19]. The main objective of this study was to develop and evaluate the feasibility of a novel speculum design. The proposed speculum aimed to facilitate visualization of the cervix without eliciting the reflexive response associated with speculum insertion. Additionally, it was designed to allow the manipulation of the cervical line by means of forceps, allowing for posterior retraction.

Material and Methods

Ethical Approval

The Scientific Ethical Committee of Kafkas University in Kars, Türkiye has granted approval for all issues pertaining to the experimental setups and evaluation methodologies under the reference number KAÜ-HADYEK 2023-047.

Animal

A total of 30 Tuj ewes, aged between 2 and 5 years, were housed under identical conditions at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm, Kafkas University, Faculty of Veterinary Medicine in Kars, Türkiye.

Experimental Design

The present investigation involved the selection of a sample of 30 animals in order to investigate the effect of CS and OS on the observation of the cervical inlet.

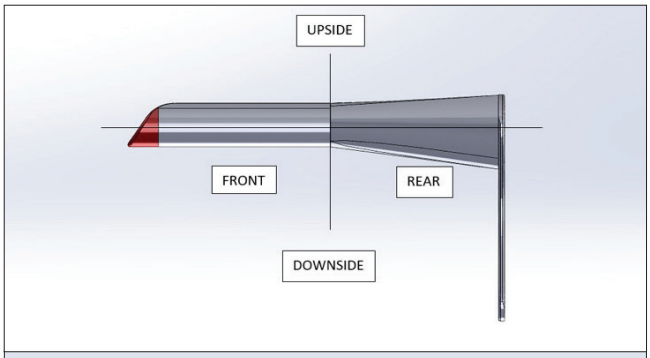

Fig 1. Parts of the Onder speculum

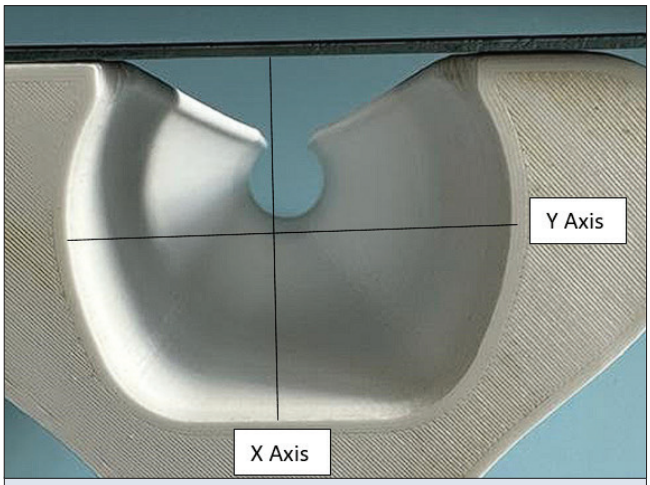

Fig 2. X and Y axis of rear cavity

To minimize the impact of individual differences, the same animals have been used in both the CS and OS experimental groups for the entire period of the research. No additional interventions were performed apart from the manipulation of the speculum.

The study also evaluated the potential efficacy of the OS for transcervical applications by evaluating its success in 10 ewes. During this phase, an assessment was conducted based on the criteria of retracting the cervical entrance and successfully completing the stages involving the removal of the speculum. The successful completion of all stages was deemed acceptable.

Speculum Design

The speculum was designed to have a length of 10 cm in the front region, 8.5 cm in the rear region, and a combined length of 18.5 cm, as depicted in *Fig. 1*. An angled transition was observed at a distance of 1.5 cm from the tip of the front portion of the speculum. To enhance structural integrity, the thickness of the material was modified to 2.5 mm in the anterior and posterior regions, while the handle portion was adjusted to a thickness of 3 mm. The anterior section was constructed in a circular shape, with a diameter of 2 cm within its inner region. The upper opening at the front measures 1 cm and the rear measures

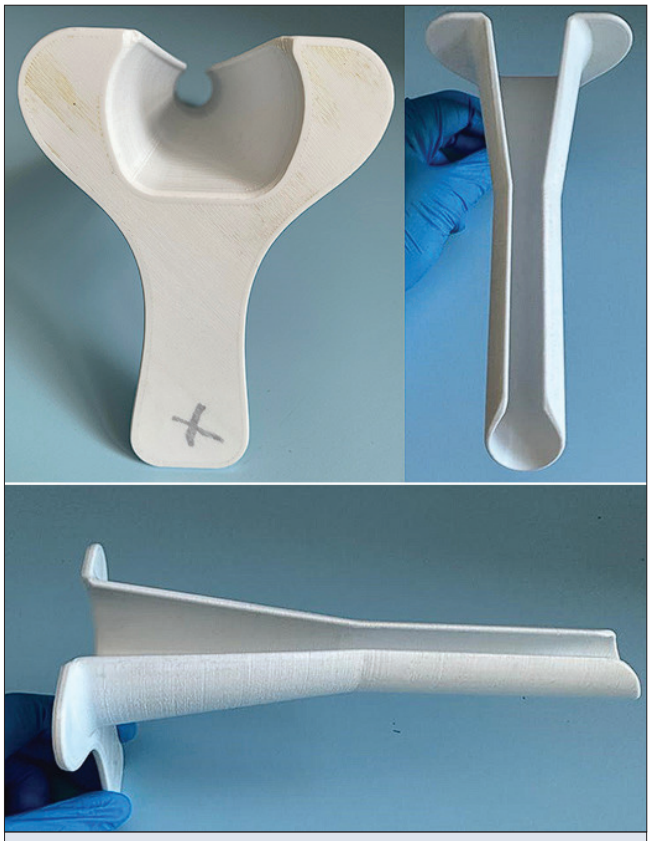

Fig 3. Printed speculum

3 cm, whereas the x and y axes have dimensions of 3.5 cm and 4.5 cm, respectively *(Fig. 2)*. The posterior portion of the speculum exhibits lateral regions measuring 2 cm, while the length of the handle is 8 cm.

The speculum was created with the computer-aided design software SolidWorks 2022, employing a three-dimensional design approach. Subsequently, it was manufactured utilizing polylactic acid material by using a Creality Ender-5 Plus 3D printer (Shenzhen, China) with a 100% fill rate. In *Fig. 3*, the printed version of the speculum is presented from different angles.

Speculum Testing and Cervical Penetration

Sheep were restrained in a standing position, and to prevent contamination, the tail was tied. Prior to the speculum application, the perineal region underwent a cleansing procedure using povidone iodine. Small CS and OS were lubricated, the vulva was opened, and speculums were inserted into the vagina in order. Subsequently, attempts were made to locate the cervical entrance simply through the use of speculum movements. A head lamp was used to observe the cervical entrance with a speculum.

At the cervical penetration phase, only the OS was used. The technique used by Pereira et al.[20] underwent modifications as described by Önder et al.[21]. The cervical entrance was grasped with forester ring forceps. Following the removal of the speculum, the cervix was subsequently moved in a caudal direction.

Table 1. *Speculums and instances of successful outcomes*

Success Status	Collin (n/ Total)	Onder (n/ Total)
Successful	8 (30)[a]	30 (30)[b]
Fail	22 (30)	0 (30)

[a,b] *Values with different superscripts in the same column are significantly different (P<0.001)*

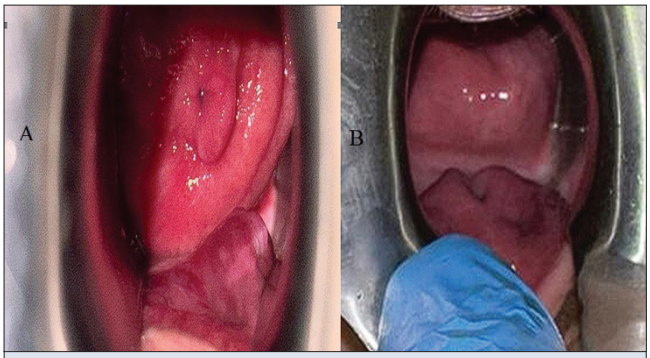

Fig 4. Successful visualization of cervical entrance (A) and Failure in visualization (B)

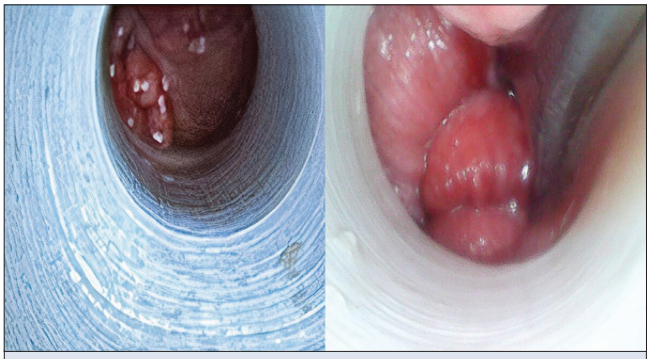

Fig 5. Visualization of two different sheep's cervical entrances with Onder speculum

Statistical Analysis

The statistical analysis of the study was conducted using the SPSS program (SPSS 28.0 for Windows; SPSS, Chicago, IL, USA). The Pearson Chi-Square method was used to analyze the data in this study. The statistical significance level has been set at an acceptable value of P<0.05, and the corresponding outcomes were reported accordingly.

Results

In this present work, it was observed that the cervical entrance was directly visible in 8 out of the 30 females examined while using the CS. With OS, cervical entrance was observed in all the animals *(Table 1)*. Statistical analysis revealed a significant difference between the speculums (P<0.001). In *Fig. 4*, successful and unsuccessful applications are presented visually with the CS. In *Fig. 5*,

a section was taken from the successful application with the OS. Regarding the retraction of the cervical entrance using the OS, our data showed a complete success in 10 randomly selected animals out of a total of 30.

The use of the OS revealed that the Polylactic acid (PLA) substance employed for producing the product using a 3D printer did not cause any adverse impacts on animals. It was observed that the OS could be easily directed into the vaginal canal, up to the opening of the cervix. The positioning of the cervical port was adjusted to the distal end of the speculum, enabling the subsequent manipulation of the cervix region using forceps. Following the secure positioning of the cervix, an assessment was conducted to determine the feasibility of removing the speculum from the vagina without encountering any complications, while the forceps remained within the speculum. During this phase, it was noted that the removal of the speculum proceeded without any complications. It has been found that in some animals, the complete insertion of the speculum into the vaginal canal is not possible. Nevertheless, it has been noted that even in such instances, the speculum did not compromise its easy-to-use features.

It was seen that the animals exhibited pushing reactions as the speculum proceeded into the vagina while using both speculums. The fact that the cervix was only visible in 8 animals when using the CS suggests that the strength of the animal's pushing reflexes might have an impact on the cervical line's position, causing it to point in the animal's dorsal region.

Discussion

In our clinical experience, the observation and manipulation of the cervix have posed significant challenges in transcervical surgeries conducted on sheep. In light of these issues encountered in visualizing the cervix predominantly through the speculum and the subsequent difficulties in stabilizing the cervical entrance, our research attempts have been directed toward designing an alternative speculum.

The utilization of computer-aided drawing tools enables the production of products through 3D printers, eliminating the need for molds in the manufacturing process. A diverse range of raw materials are utilized in the manufacturing process of items through the utilization of 3D printers. According to reports, the commonly utilized raw materials consist of filaments, which are thermoplastic polymers. PLA, a type of thermoplastic material, is notable for its biocompatible and biodegradable characteristics, as well as its comparatively reasonable pricing [22,23]. It finds applications in diverse industries, including the automotive, textile, and biomedical sectors [23]. In the presented study,

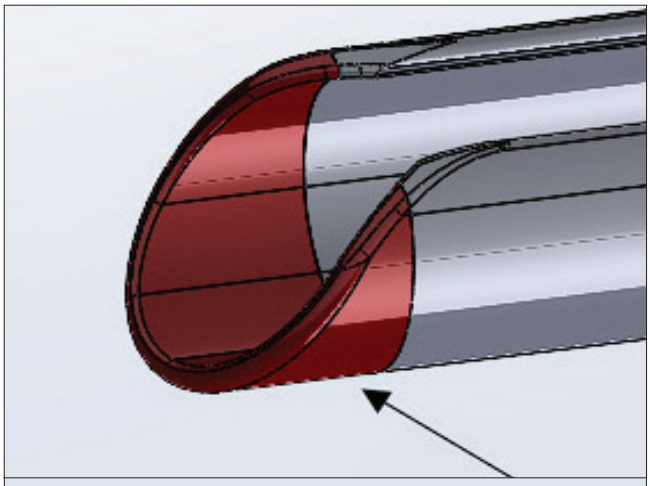

Fig 6. Arrow indicates the area X

PLA was preferred in speculum production due to its use in the biomedical field. In our investigation, we did not see any discernible adverse effects of this substance utilized in the fabrication of speculums on the animal subjects. No adverse outcomes, such as stretching, bending, or breaking of the speculum, were seen throughout usage, as the material was manufactured to meet the specified thickness and full filling rate outlined in its design.

The OS, derived from the duck-billed speculum and the CS, has a total length of 18.5 cm. The design of the instrument took into consideration the need to eliminate sharp edges. The front section of the speculum aims to ensure that, following the insertion of the device into the vagina, any manipulations performed in the cranial vaginal region do not cause the cervical entrance to move farther cranially. The part of the speculum that will provide the initial entrance into the vagina is shaped with a backward inclination to prevent tissue damage and facilitate a smoother transition. Also, another crucial objective at this stage was to ensure that the cervical entrance is positioned within the X area *(Fig. 6)* once it has been observed.

The speculum is specifically engineered to have a 2 cm diameter in its anterior 10 cm segment, facilitating the introduction of the Forrester ring forceps. Additionally, a 1 cm opening is strategically positioned in the ventral area of the speculum to provide easy removal without any interference with the forceps while it remains inside the vagina. It was hypothesized that an increased aperture size of 1 cm could potentially result in the accumulation of vaginal tissue within the speculum, hence limiting the intended procedures. The presence of a closed lower region and a c-shaped design in the 10 cm part of the speculum effectively prevents the entry of vaginal tissue. The findings of this study demonstrate that the OS effectively maintains the position of the cervical line and aligns it with the vulva, therefore validating the inclusion of these features in the design of the speculum.

In the posterior section of the speculum, the upper aperture increases from 1 cm to 30.00 mm. Simultaneously, the length of the x-axis undergoes continuous expansion, ultimately reaching a final measurement of 45.00 mm. The utilization of forceps has been effectively manipulated by the augmentation of the X and Y axes. The design of the speculum includes a flat base in the posterior region, which serves the purpose of providing less covering within the vaginal canal and facilitating ease of application. The lateral regions of the speculum, similar to the Collin, were designed specifically to minimize the risk of contamination along the inner surface of the instrument during its use.

The current investigation revealed significantly limited efficacy of the CS in spotting the cervical opening without any form of manipulation. It was thought that this was due to the fact that the CS was not designed for use in assisted reproductive techniques for sheep. The proper positioning of the cervical entrance with the newly constructed speculum demonstrates agreement with the aforementioned idea. According to available reports, it has been observed that Allis forceps and Pozzi forceps are frequently used in research conducted using the CS [9,24]. The utilization of Forrester ring forceps has been favored due to the fact that the use of Pozzi forceps may result in minor instances of hemorrhaging, as observed in our investigations [21]. Based on our research findings, the utilization of Pozzi forceps facilitates the extraction of the cervical line in a more efficient manner due to their ability to pierce and pull the cervical entrance area. Conversely, the use of Forrester ring forceps presents challenges in extracting the cervical line without causing active bleeding. The utilization of the OS facilitates the manipulation with the Forrester ring forceps due to the repositioning of the cervical entrance in a cranial direction, resulting in increased ease of maneuverability and a degree of resistance.

In summary, our study determined that the newly developed speculum effectively supported the implementation of non-surgical assisted reproductive procedures in small ruminants. Furthermore, it is believed that the use of the OS holds potential for assisting researchers in their pursuit of advancing non-invasive reproductive techniques in small ruminants.

Data Availability

The data used to support the findings of this study are available from the corresponding author (N.T. Önder) upon request.

Ethical Approval

The Scientific Ethical Committee of Kafkas University in Kars, Türkiye has granted approval for all issues pertaining to the experimental setups and evaluation methodologies under the reference number KAÜ-HADYEK 2023-047.

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We would like to extend our heartfelt gratitude to Salman Mammadli for his invaluable assistance in rendering the speculum through the use of the Solidworks software, then producing it via a 3D printer.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution Section

NTO designed the speculum and the study. NTO performed the experiment. NTO wrote the manuscript. TG, MCK, OS, MCD, CK, SY, YÖ provided assistance during the experiment. NTO performed the statistical analysis.

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Research Article

Evaluation of the Impact of Long-term Treadmill Exercise on Antioxidant Capacity and Immune Function in Mice

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Abstract

Regular exercise is known to confer various health benefits, but the specific impact of exercise intensity on antioxidant capacity and immune function remains complex and incompletely understood. Our study investigates the impact of exercise intensity on antioxidant capacity and immune function in mice. Mice were subjected to various exercise intensities (low, moderate, high) on a treadmill. Antioxidant enzyme activity, oxidative stress markers, ROS production, hemoglobin, hematocrit, leukocyte counts, lymphocyte subsets, and phagocytic activities were assessed. Exercise intensity influenced antioxidant enzyme activity, with low and high intensities showing significant effects. High-intensity exercise led to increased lipid peroxidation but improved the glutathione redox ratio. Exercise, irrespective of intensity, significantly affected immune function, notably increasing leukocyte counts, T-cell and B lymphocyte subsets, and phagocytic activities. Even at lower intensities, exercise profoundly impacts antioxidant capacity and immune function. These findings highlight the relationship between exercise, oxidative stress, and immune modulation.

Keywords: Antioxidant activity, Immune response, Mice, Treadmill exercise

Introduction

Physical exercise contributes to a healthy lifestyle by improving cardiovascular fitness, muscle strength, and weight management [1]. Recent research has increasingly focused on the broader implications of exercise, including its potential impact on the body's ability to combat oxidative stress and bolster immune defenses [2,3]. Adwas et al.[4] suggested that oxidative stress occurs when there is an imbalance between the production of harmful reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. Prolonged oxidative stress can lead to cellular damage and contribute to the development of various diseases, including cancer and cardiovascular disorders. Antioxidants play a crucial role in mitigating the harmful effects of oxidative stress [5-9].

The immune system is a complex network of cells and molecules that defend the body against infections and diseases. An efficient immune system is vital for maintaining overall health and well-being [10]. The potential link between exercise, antioxidant capacity, and immune function is an area of growing interest in scientific research.

Long-term treadmill exercise in mice has been the subject of extensive research with significant findings regarding its effects on antioxidant capacity and immune function [11-13]. Research has indicated that prolonged treadmill exercise increases the body's various antioxidants [14]. These antioxidants, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, are crucial in neutralizing harmful reactive oxygen species (ROS), thus mitigating oxidative stress.

Exercise has been associated with a reduction in markers of oxidative stress. The decrease in oxidative stress markers encompasses a lower concentration of ROS and a decrease in lipid peroxidation products [15]. Such a reduction is vital as it helps prevent cellular damage and mitigates the risk of numerous chronic conditions linked to oxidative stress. Moreover, long-term treadmill exercise has positively impacted immune function [16]. Studies have observed that exercised mice exhibit improved immune responses

when subjected to specific antigens or pathogens [17]. This enhancement suggests that exercise may bolster the ability of the immune system to mount effective defenses against infections. Exercise triggers a cascade of physiological responses contributing to improved antioxidant capacity and immune function [14,18]. Regular exercise promotes mitochondrial biogenesis, enhancing cellular energy production. This increase in mitochondrial activity can enhance the body's capacity to neutralize ROS and reduce oxidative stress.

Exercise also promotes the release of anti-inflammatory cytokines, which can modulate the immune response. This modulation is essential for ensuring that the immune system functions optimally, responding effectively to threats while avoiding excessive inflammation, which can be harmful [3]. Furthermore, exercise induces the release of endorphins, which enhance mood and immune regulation. These chemicals positively influence the immune system's activity. In addition to these mechanisms, exercise promotes better circulation, ensuring that antioxidants and immune cells are efficiently transported throughout the body [19]. Improved blood flow facilitates the delivery of these essential components to areas where they are needed most.

Long-term treadmill exercise initiates a series of interconnected events culminating in increased antioxidant capacity and enhanced immune function in mice. Understanding these mechanisms is crucial for advancing our knowledge of exercise physiology and potentially translating these findings into strategies that promote better human health and well-being. Our study focuses on the impact of long-term treadmill exercise on antioxidant capacity and immune function in mice. Previous research has consistently shown that sustained exercise can enhance antioxidant levels, reduce oxidative stress, and improve immune responses in these animals. Complex mechanisms drive these improvements, including increased mitochondrial activity, the release of anti-inflammatory cytokines, and endorphin's influence. These processes collectively underpin the observed benefits of exercise on antioxidant defenses and immune function.

Our primary objective is to investigate the physiological changes caused by long-term treadmill exercise and understand the underlying mechanisms. It may inform strategies to improve health and resilience against oxidative stress and infections in mice.

Material and Methods

Ethics

The Ethics Committee of Hebei Provincial Hospital approved our study and experimental techniques and conducted according to the ethical guidelines on animal experiments in China (Approval no. NK20221008A07)

Animals

Our study acquired 48 three-week-old Kyoto Wistar rats, consisting of 24 males and 24 females, at the start of the experiment. We chose these rats at their current age due to their rapid growth rate, and we estimated that they would be adults at the end of our study, making it suitable for comparative analyses. Moreover, nutritional interventions during this period were associated with sensitive changes in the immune system.

Then, animals were kept in cages (4 per cage) at climate-controlled conditions of 23ºC temperature, 50% humidity, and a day/night cycle of 12 h. Furthermore, they were subjected to a standard diet and supplied with water and food ad libitum. The animals were weighed at the end of every week, and the ponderal homogeneity index was determined and recorded. After the first ten days of acclimatization to these laboratory conditions, the animals were randomly assigned to one of the four experimental groups, with each group consisting of 12 animals; the sedentary group consisted of the non-exercising group that was sacrificed after 28 weeks of training; the low-intensity exercise group was sacrificed at 32 weeks of training; the moderate intensity exercise group consisted were sacrificed at 48 weeks of training and the high-intensity exercise training group were sacrificed at 54 weeks of training.

Exercise Training Program

All animals were subjected to physical exercises at eight weeks of age *(Fig. 1)*. The aerobic treadmill exercise consisted of running on the treadmill for 40 minutes per day, seven days a week. The maximal speed was set to 80% during the 28-, 32-, 48- and 54-week training sessions. The treadmill was inclined at 0º for all experimental groups. At the optimum speed of the treadmill, a speed test was carried out to determine the average running speed of the animals. The exercises consisted of a warm-up on the treadmill for 10 min at 15 m per min, after which the speed was increased gradually to 8 m/min every 60 sec until the animals could not maintain the treadmill belt's pace.

The maximal running speed was estimated as the last speed recorded for at least 60 sec, and the initial weeks of training allowed the animals to adapt to the training exercise. The adaptation was set so every animal could attain the maximum speed before performing subsequent exercises. In the subsequent training exercises, the running speed was slowly increased until the maximal speed was attained every four weeks while maintaining the intensity at 80%. Animals in the sedentary group were placed on a

stationary treadmill to have the same handling effect as those in other exercising groups.

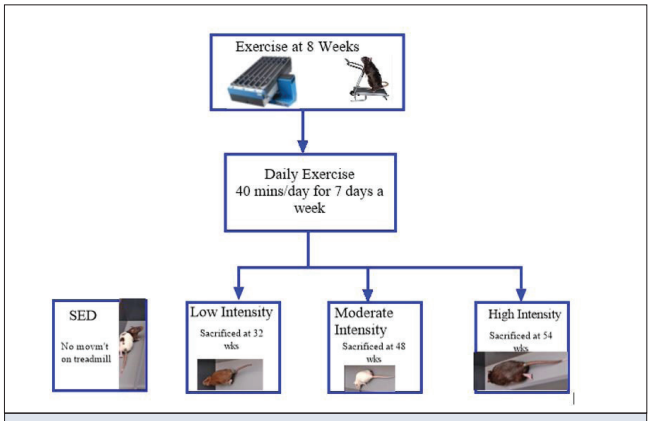

Fig 1. Illustration of the exercise training program

Sample Collection

All animals were anesthetized using intraperitoneal injections of Xylazine and Ketamine at 5 mg/kg of body weight before exsanguination to collect blood samples and tissues. The samples were collected at 28 weeks, 32 weeks, 48 weeks, and 54 weeks. The blood, liver, spleen, and peritoneal macrophages were harvested and processed. The blood was extracted from the heart tissues and ethylenediaminetetraacetic (EDTA) anticoagulated tubes. The EDTA blood samples facilitated the determination of the number of leukocytes, hematocrit, and hemoglobin. Moreover, they were used in characterizing the composition of blood lymphocytes. The liver and peritoneal macrophages were frozen to analyze posterior oxidative stress.

Determining Oxidative Stress

Two grams of frozen liver samples were added to 15% (w/v) of ice-cold phosphate buffer (30 mM, pH = 7.0) before mixing for 5 min to achieve a homogenous mixture. The mixture was applied to different centrifugation cycles at 5ºC: 2000x g for 15 min, 10000x g for 20 min, and 16000x g for 30 min. After that, the supernatant was obtained to estimate the level of antioxidant enzymes and reduce the levels of glutathione and oxidized glutathione ratios. Lipid peroxidation was determined using pellets extracted from the mitochondrial fraction during the initial phase of the centrifugation process. The biuret technique and bovine serum albumin were used to determine the total protein content of the supernatant and the pellets. All antioxidant enzyme assays were performed at room temperature.

Superoxide dismutase (SOD) activity was evaluated spectrophotometrically at 560 nm using the xanthine-xanthine oxidase system. The assays were performed in a modified potassium phosphate buffer (40 mM, pH 7.0) containing EDTA (1.5 mM), hypoxanthine (12 mM), nitroblue tetrazolium chloride (NBT, 12 mM), and approximately 0.3 mg of protein from the enzymatic extract. Catalase (CAT) activity was determined polarographically using a Clark-type oxygen electrode. The reaction occurred in a modified potassium phosphate buffer (45 mM, pH 7.2) with hydrogen peroxide (9.5 M). 0.35 mg of protein from the samples was added to initiate the reaction. Glutathione peroxidase (GPx) activity was assessed spectrophotometrically at 340 nm. The assay involved a modified potassium phosphate buffer (90 mM, pH 7.2), EDTA (1.2 mM), reduced glutathione (GSH, 110 mM), glutathione reductase, NADPH (11 mM), and 0.35 mg of protein from the enzymatic extract. Glutathione reductase (GR) activity was measured spectrophotometrically at 340 nm under magnetic stirring. The reaction system consisted of a modified potassium phosphate buffer (95 mM, pH 7.0), EDTA (0.6 mM), NADPH (11 mM), and 1.4 mg of protein from the enzymatic extract. The addition of oxidized glutathione (GSSG, 120 mM) initiated the kinetic reaction.

Nonspecific lipid peroxidation (LOP) levels were assessed by quantifying lipid peroxides as thiobarbituric acid reactive substances (TBARS). The hepatic lipid extract was combined with 1 mL of thiobarbituric acid (TBA) reagent (0.32% (m/v) TBA, 35% (m/v) trichloroacetic acid (TCA), and 0.012% (m/v) butylated hydroxytoluene (BHT)). This mixture was heated at 95°C for 12 min and then rapidly cooled by immersion in an ice bath. Subsequently, the mixture was centrifugated at 1500× g for 8 min, at 5°C, and the resulting supernatant was collected for further analysis.

Determination of Phagocytic Activity

The process was conducted through flow cytometry analysis, focusing on blood monocytes and granulocytes, using the PhagotestTM kit. Initially, blood was incubated with fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* for 8 minutes at 38°C. Subsequently, the samples were placed on ice, and a quenching solution was added to halt phagocytosis. After a washing step to remove excess reagents, erythrocytes were lysed. Following DNA staining, data acquisition was performed using the Gallios™ Cytometer, and subsequent analysis was conducted utilizing FlowJo v. ten software. Our analysis employed forward-scatter and side-scatter characteristics to select monocyte and granulocyte populations. The quantification of phagocytic monocytes and granulocytes relied on assessing the percentage of FITC+ cells. Additionally, we measured their phagocytic activity using the mean fluorescence intensity (MFI). In each experiment, we compared the proportion of phagocytic cells and their relative phagocytic activity to that of the sedentary (SED) group, with the data from sedentary rats serving as the reference point at 100%.

Lymphocyte Composition in Blood

Blood lymphocyte subsets were identified after erythrocyte osmotic lysis using specific antibodies (mouse anti-rat NKR-P1A, CD45RA, TCRαβ, or TCRγδ) conjugated to FITC. Cells were incubated with saturating antibody concentrations (4°C, 20 min), followed by fixation (0.5% p-formaldehyde) and storage (4°C) until flow cytometry analysis. Each cell sample included a blank control. Data were collected using a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA). Blood lymphocytes were quantified as subset counts, considering the lymphocyte number from the hematology analyzer and the subset percentages obtained via flow cytometry.

Statistical Analysis

All analyses were carried out in GraphPad Prism version 9.5.1. Descriptive statistics were presented as mean ± SD (standard deviation) for the four experimental groups. We assessed the equality and normality of the results using Levene's and Shapiro-Wilk's tests, respectively. Once these conditions were confirmed, we applied a one-way ANOVA test to evaluate parameters such as phagocytic monocyte and granulocyte proportions, phagocytic activities, and ROS production. In cases where significant differences were observed, we performed Bonferroni's post hoc test to make specific group comparisons.

Variables that did not meet parametric assumptions, such as hemoglobin concentration, hematocrit, number of leucocytes, lymphocytes, monocytes, and granulocytes in the blood, were analyzed using non-parametric tests such as the Kruskal-Walli's test followed by the Mann-Whitney U test to determine significance.

We conducted a repeated measures ANOVA test to analyze time-dependent parameters, including body weight (BW). When comparing two groups, such as males and females, or performance within training, we used unpaired or paired Student's t-tests as needed. We applied Pearson or Spearman correlation tests to assess correlations between variables, considering data normality and equality. We considered differences significant when $P<0.05$.

Results

Baseline Parameters of Body Weight, Food, and Water

We minimized animal stress by measuring animals' body weight, food, and water consumption per week to comprehend their general conditions during the exercise. *Table 1* shows the initial Weight, final Weight, ponderal gain, and average liver weight of all animals in the four experimental groups.

Table 2 displays a standard comparison of the Mean food and water consumption at the experimental protocol's beginning and end.

Performance of Training Groups

Our study evaluated the performance of males and females across the four groups during the training programs. The final exhaustion tests revealed no significant differences between males and females based on the highest distance covered *(Fig. 2)*. A visual analysis of the animals while on the treadmill did not reveal significant differences based

Table 1. *Ponderal homogeneity index (PH), initial and final animal body weights (g), ponderal gain (PG), and mean relative liver weight for all experimental groups*

Groups	PH	Initial Weight (g)	Final Weight (g)	PG	Average Liver Weight (g)
SED	0.53	152.7±12.4	461.7±25.8$^{\dagger,\&}$	0.55±0.03$^{\dagger,\&}$	0.014±0.03
Low Intensity	0.46	156.3±12.2	394.3±12.5$^{*,\&}$	0.50±0.03$^{*,\&,\#}$	0.017±0.01$^{\&,\#}$
Moderate Intensity	0.53	142.9±12.4$^{*,\dagger,\#}$	536.2±11.6$^{*,\dagger,\#}$	0.64±0.02$^{*,\dagger,\#}$	0.010±0.05
High Intensity	0.45	145.6±10.65	429.6±27.7$^{\&}$	0.55±0.02$^{\dagger,\&}$	0.013±0.02

*The values are presented as means ± SD. * P<0.05 compared with SED; † P<0.05 compared with Low Intensity; & P<0.05 compared with moderate intensity; # P<0.05 compared with high intensity; SED (Sedentary Group)*

Table 2. *A standard comparison of the Mean food and water consumption at the experimental protocol's beginning and end*

Groups	Initial Food Consumption (g)	Final Food Consumption (g)	Initial Water Consumption (g)	Final Water Consumption (g)
SED	15.4±0.1	16.2±2.6	20.1±1.4	15.3±2.6
Low Intensity	14.5±5.1	18.4±0.6	19.9±0.5	22.8±1.4$^{\&,*}$
Moderate Intensity	13.9±0.5	18.6±2.6	18.6±0.8	15.1±0.3
High Intensity	14.9±0.8	16.7±0.7	16.3±1.5$^{\&,\dagger,*}$	19.9±1.3

*The values are means ± SD. * P<0.05 compared with SED; † P<0.05 compared with Low Intensity; & P<0.05 compared with moderate intensity, # P<0.05 compared with high intensity; SED (Sedentary Group)*

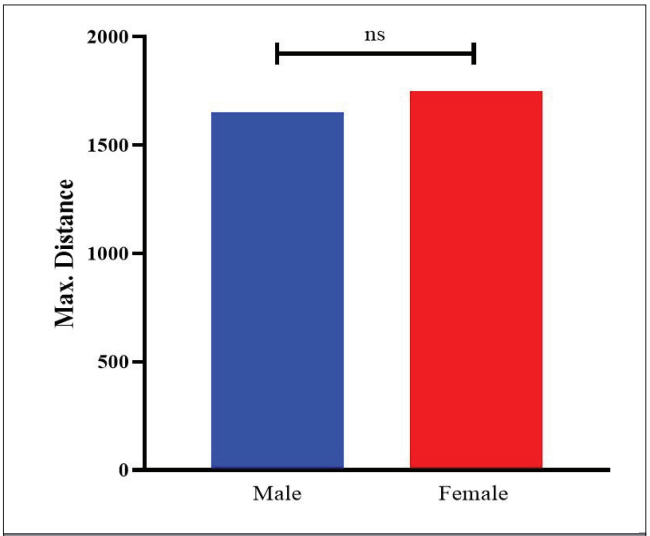

Fig 2. Effect of exercising on the treadmill on the final exhaustion tests

on gender and groups on the level of adaptation to the treadmill.

Antioxidants

Table 3 displays hepatic antioxidant enzyme activity.

Table 4 displays oxidative stress markers: LPO and the GSH/GSSG ratio.

Production of ROS by Macrophages

We analyzed the production of ROS by peritoneal macrophages and showed that the high-intensity exercise group generated the highest levels of ROS compared to other groups *(Table 5)*. Moreover, the ROS was higher in group 3 compared to group 2 ($P<0.05$) and group 2 compared to group 1 ($P<0.05$). These differences in the levels of ROS were statistically significant ($P<0.01$).

Table 5 presents ROS production at 40 and 80 min.

Concentration of Haemoglobin, Haematocrit, and Leukocyte after Exercise

We evaluated the hemograms of experimental groups to identify the effects of exercise intensity on white and red blood cell levels. Our findings showed a significant increase in hemoglobin (HGB) and hematocrit (HCT) in the low-intensity group compared to the sedentary group. Moreover, there were statistically significant differences associated with a decrease in HGB and HCT between groups 3 (moderate intensity) and 4 (high intensity) in comparison to group 1; the levels of HGB and HCT significantly reduced ($P<0.05$) *(Fig. 3)*.

Our analysis of the count of white blood cells *(Fig. 4)* showed that in the high-intensity group, there was two-fold increase in the number of leukocytes, monocytes, and lymphocytes compared to the sedentary group ($P<0.05$).

Table 3. *The activity of hepatic antioxidant enzymes*

Antioxidant Enzymes	SED	Low Intensity	Moderate Intensity	High Intensity
SOD (U.min^{-1}.mg^{-1} protein)	2.19±0.18	2.76±0.11*	2.36±0.21†	2.69±0.04*
CAT (mmol H_2O_2.min^{-1}.mg^{-1} protein)	0.41±0.051	0.48±0.06	0.15±0.010*,†	0.25±0.03*,$^{\dagger,\&}$
GPx (µmol NADPH oxidized.min^{-1}.mg^{-1} protein)	419.4±22.0	353.7±30.6*	371.9±40.9*	332.4±22.6*
GR (µmol NADPH oxidized.min^{-1}.mg^{-1} protein)	30.44±2.33	24.58±0.71*	20.89±2.36*,†	19.73±0.13*,†

*The values are means ± SD (n = 7), with two replicates. * P<0.05 compared with SED; † P<0.05 compared with Low Intensity; & P<0.05 compared with moderate intensity, # P<0.05 compared with high Intensity; SED (Sedentary Group), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR)*

Table 4. *Oxidative stress markers: lipid peroxidation (LPO) and ratio GSH and GSSG*

Oxidative Stress Markers		SED	Low Intensity	Moderate Intensity	High Intensity
LPO (µM MDA. mg^{-1} protein)	Total Fraction	0.053±0.02	1.16±0.26*	0.61±0.11*,†	0.53±0.11*,†
	Mitochondrial Fraction	0.41±0.14	1.24±0.26*	0.53±0.06†	0.33±0.91†
GSH/GSSG		3.06±0.25	2.68±0.16	3.67±0.91	4.69±1.19*,†

*The values are means ± SD, with two replicates. * P<0.05 compared with SED; † P<0.05 compared with Low Intensity; & P<0.05 compared with moderate intensity, # P<0.05 compared with high intensity; SED (Sedentary Group)*

Table 5. *Production of ROS after 30 and 60 mins*

Time	SED Group	Low Intensity	Moderate Intensity	High Intensity
40 min	5773±934.6	9915±1122.2*,#	10620±986.4#	11087±1001.2*,#
80 min	7026±837.1	10593±1329.1*,#	12587±1255.1#	13004±1342.2*,#

*The values are means ± SD (n = 7), with two replicates. * P<0.05 compared with SED; # P<0.05 compared with high intensity; SED (Sedentary Group)*

Similarly, there was significant increase in groups 3, and 2 compared to group 1 (P<0.05). There were no significant changes in the number of granulocytes across the four groups (P<0.05).

We analyzed the major subsets of lymphocytes in the blood of animals in each group *(Fig. 5)*. There were statistically significant differences in the number of T-cells between the high-intensity and sedentary groups (P<0.05). Moreover, the moderate-intensity and low-intensity groups showed a significant increase in the count of T cells and B lymphocytes (P<0.05).

The effects of exercise training on phagocytic activities showed a statistically significant increase from the sedentary group (baseline) to the high-intensity group *(Fig. 6)*. The baseline percentage of blood phagocytic monocytes and granulocytes was 45% (group 1), which was used as a reference compared to other groups. We noted an increase of 170% within 36 hours of completing the tests in group 4 (P<0.01), 140% after completing tests in group 3 (P<0.01), and 115% after completing tests in group 1 (P<0.01).

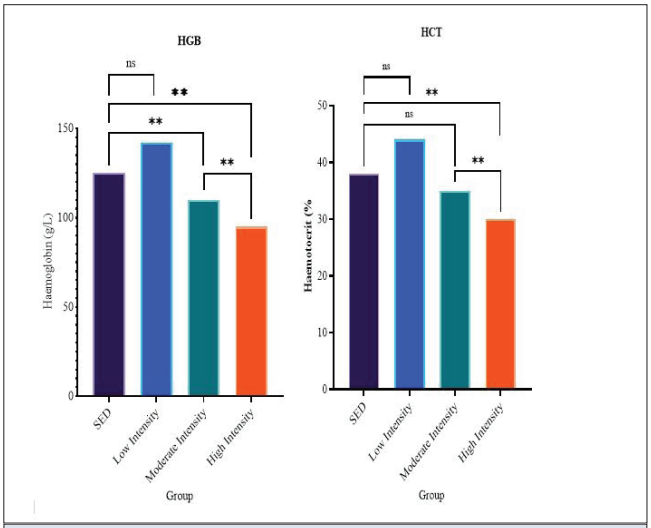

Fig 3. Haemoglobin, Haematocrit, and Leukocyte concentration after exercise, **P<0.05

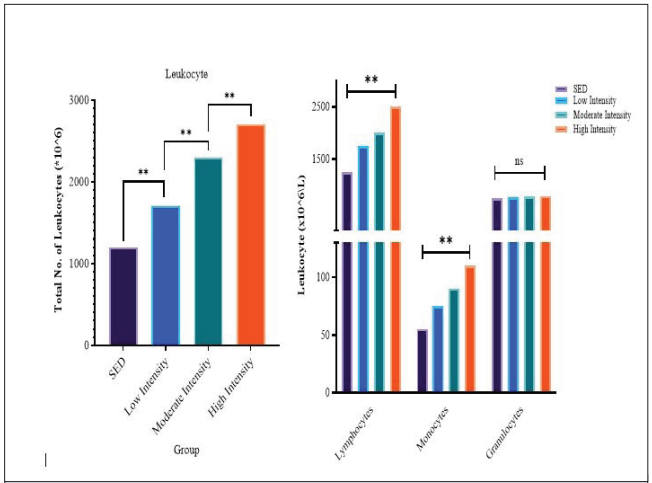

Fig 4. Analysis of the variations in the number of leukocytes per liter, ** P<0.05

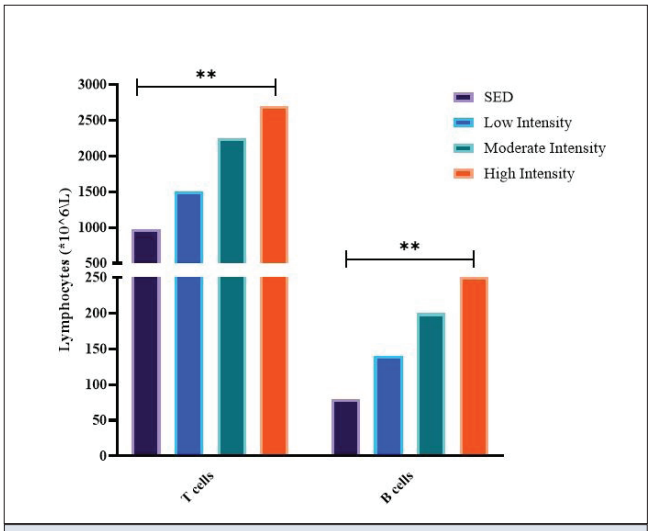

Fig 5. The variations in the levels of lymphocytes across the different groups, ** P<0.05

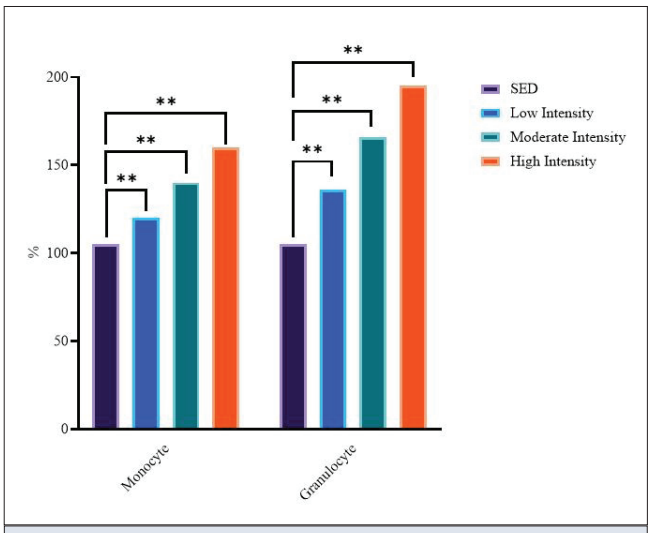

Fig 6. Changes in phagocytic monocytes and Granulocytes across the four groups, ** P<0.05

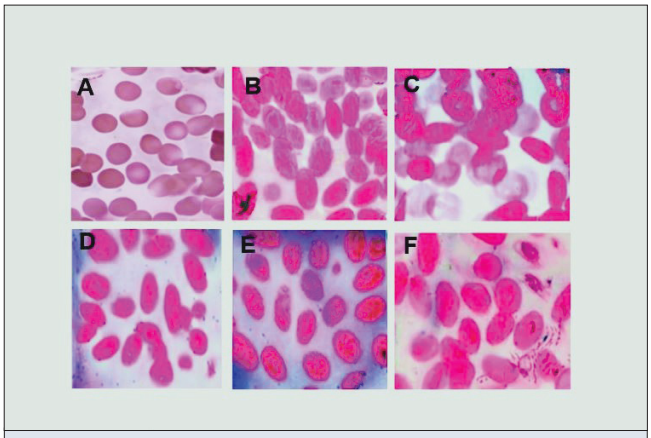

Fig 7. Circulating Lymphocytes across the experimental groups

According to *Fig. 7*, Section A shows the circulating lymphocytes after smearing with Wright's stain, (B) heterophils, (C) Large and small lymphocytes, (D) Erythrocytes and Monocytes, (E) Eosinophils, (F) Basophils. They have quiescent circulation with significantly reduced cytoplasm. The entire cytoplasmic volume is filled with the nucleus whose chromatin is compact and coiled with varying levels of density.

Discussion

Our study investigated the impact of various treadmill exercise intensities on antioxidant enzyme activity, oxidative stress markers, and ROS production in mice. Low and high-intensity exercise increased hepatic antioxidant enzyme activity, specifically in SOD and CAT, while moderate-intensity exercise showed variable effects. Conversely, all exercise intensities resulted in reduced GPx and GR activities. Low and high-intensity exercise groups exhibited elevated lipid peroxidation (LPO), indicating oxidative damage. In contrast, high-intensity exercise positively influenced the GSH/GSSG ratio, suggesting a more reduced cellular environment. ROS production was consistently higher in exercise groups compared to sedentary mice, regardless of intensity.

Our findings were consistent with Ghane et al.[20], who proposed that the increased hepatic antioxidant enzyme activity in SOD and CAT, with low and high-intensity treadmill exercise, can be attributed to the body's response to increased oxidative stress during exercise. Similarly, Thirupathi et al.[15] showed that exercise generates ROS as a natural byproduct of increased metabolic activity. To counteract the potential damage caused by these ROS, the body upregulates the production of antioxidant enzymes like SOD and CAT. SOD converts superoxide radicals into hydrogen peroxide, while CAT neutralizes hydrogen peroxide into water and oxygen. The elevated enzyme activity observed manifests the adaptive response to exercise-induced oxidative stress aimed at preserving cellular integrity.

Valado et al.[21] postulated that the reduction in hepatic glutathione peroxidase (GPx) and glutathione reductase (GR) activities following exercise reflects a more complex interplay of molecular mechanisms. GPx and GR are critical components of the glutathione redox cycle, detoxifying hydrogen peroxide and maintaining cellular redox balance. Exercise-induced changes in GPx and GR may result from adaptations to decreased hydrogen peroxide levels or a shift in alternative pathways utilization during exercise [22]. These alterations in enzyme activity could represent an adaptive response to maintain redox homeostasis in the face of increased exercise-related demands. However, further research is required to elucidate the precise molecular mechanisms at play.

The elevated LPO observed in low and high-intensity exercise groups indicates oxidative damage to cell membranes. Zhou et al.[23] showed that increased metabolic activity generates more ROS during exercise, which can target cellular lipids. ROS can initiate chain reactions, leading to the peroxidation of lipids, ultimately compromising cell membrane integrity. The higher LPO levels suggest that exercise intensity influences the extent of oxidative damage. However, the precise molecular pathways linking exercise intensity to LPO levels and the modulation of lipid peroxidation require further investigation. Our findings aligned with Alizadeh et al.[24], who showed that the high-intensity exercise group's improved glutathione redox ratio (GSH/GSSG) suggests a more reduced cellular environment, this ratio reflects the balance between reduced (GSH) and oxidized (GSSG) forms of glutathione, a vital cellular antioxidant. High-intensity exercise may stimulate the synthesis of GSH or enhance its recycling, reducing GSSG levels and elevating the GSH/GSSG ratio. This molecular response improves cellular redox balance during high-intensity exercise, potentially mitigating oxidative stress.

The higher ROS production in exercise groups at both 40 and 80 min is an expected outcome of increased metabolic activity during physical exertion. Exercise elevates oxygen consumption, leading to more significant ROS generation in mitochondria, the primary site of ROS production [25,26]. Molecular mechanisms behind this phenomenon involve increased electron transport chain activity, which can leak electrons and generate ROS. Furthermore, releasing pro-inflammatory cytokines during exercise can stimulate immune cells to produce ROS. These molecular events collectively contribute to higher ROS levels during exercise, highlighting the complex relationship between physical activity and oxidative stress.

Our study showed that high-intensity exercise significantly increased T-cell counts and phagocytic activities, indicating a robust immune response. Even moderate and low-intensity exercise led to notable increases in T-cells, B lymphocytes, and phagocytic activities, underscoring the immune-boosting potential of physical activity. Exercise can stimulate the release of stress hormones, such as cortisol and catecholamines (e.g., epinephrine), as part of the body's "fight or flight" response. These hormones play a pivotal role in mobilizing immune cells. For instance, cortisol can trigger the release of stored T-cells from the spleen and lymph nodes into the bloodstream [27,28]. T-cells are key players in cell-mediated immunity and recognize and target infected or abnormal cells [29]. The increase in T-cell counts suggests that high-intensity exercise can stimulate the proliferation or mobilization of T-cells, potentially enhancing the body's ability to mount an immune response against pathogens or aberrant cells [30].

Similarly, catecholamines can enhance the trafficking of immune cells, including B lymphocytes, by increasing their adhesion to blood vessel walls and promoting their migration to areas of potential infection [31,32]. This hormonal response contributes to increased T-cell and B lymphocyte counts. The elevated counts of both T-cells and B lymphocytes suggest that exercise, irrespective of intensity, can enhance both arms of the immune system. The molecular mechanisms driving these changes may involve releasing factors like interleukins and growth factors, which support the proliferation and activation of immune cells.

Exercise-induced inflammation is a crucial driver behind the increased phagocytic activity of immune cells. Zhou et al.[33] suggested that physical activity can lead to the release of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). These cytokines act as signaling molecules that alert immune cells to potential threats. They also enhance the phagocytic abilities of monocytes and granulocytes. IL-6, for instance, can stimulate the production of acute-phase proteins, including C-reactive protein (CRP), which can bind to pathogens and facilitate their engulfment by phagocytes. Additionally, TNF-α can activate immune cells, making them more efficient at phagocytosis. Combining these cytokine-driven processes amplifies phagocytic activity, helping the body defend against invading micro-organisms [33].

Exercise-induced changes in hemoglobin levels can be linked to the production of erythropoietin (EPO), a hormone that regulates red blood cell production. Similarly, Tomczyk et al.[34] showed that during exercise, there is an increased demand for oxygen transport to active muscles. This stimulates the kidneys to release EPO, which, in turn, promotes the production of red blood cells in the bone marrow. These newly formed red blood cells are rich in hemoglobin, the oxygen-carrying protein. The increase in hemoglobin levels observed in response to exercise reflects this adaptive response aimed at improving oxygen-carrying capacity to meet the heightened oxygen demands of working muscles.

In conclusion, our study found different responses to exercise, affecting antioxidant capacity and immune function. Exercise intensity influenced antioxidant enzyme activity, with low and high intensities demonstrating significant effects. However, exercise resulted in increased lipid peroxidation, especially at high intensity. The glutathione redox ratio improved at high intensity, indicating better redox balance. Immune function had dynamic responses with exercise, even at lower intensities, significantly affecting leukocyte counts, subsets of lymphocytes, and phagocytic activities. These findings highlight the complex interaction between exercise, oxidative stress, and immune modulation. High-intensity exercise emerged as a robust inducer of immune responses, while even moderate and low-intensity exercise showed substantial effects. These findings provide valuable information for optimizing exercise regimens to promote overall health and immune resilience. Further research into the underlying molecular mechanisms is essential for a deeper understanding of these responses.

Availability of Data and Material

The data presented in this study are available on request from the corresponding author (X. Liu).

Acknowledgment

We are thankful to Ding Yi and Huang Feihong, the heads of the animal department, for their assistance in the research process.

Ethical Approval

The Ethics Committee of Hebei Provincial Hospital approved our study and experimental techniques and conducted according to the ethical guidelines on animal experiments in China (Approval no. NK20221008A07)

Financial Support

None.

Conflict of Interest

The Authors declared that there is no conflict of interest.

Authors Contribution

Conceptualization and Data Collection: X.L.; Methodology, Validation, and Writing-original draft preparation: X.W.; Writing-review and editing with Financial Support: T.W. All authors have read and agreed to the published version of the manuscript.

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Short Communication

Molecular Epidemiology of Rabies in the Eastern and Southeastern Anatolian Regions of Türkiye, 2016-2021

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Abstract

Rabies can be transmitted through biting, contact of infected saliva with damaged skin, and high levels of aerosol exposure. Dog and fox-mediated rabies are endemic in Turkey. In this study, the N gene and the G-L intergenic region sequences were obtained from rabies-positive brain samples from Eastrern and Southeastern Anatolia (2016-2021). These sequences were analyzed with sequences available in GenBank. They were used to create phylogenetic trees, demonstrating that all sequences from this study belong to the three different subclades (ME1a, ME2 and, CA2) of Cosmopolitan clade. This study provides a better understanding of the molecular epidemiology of rabies virus in the mentioned regions will have benefits for continuing comprehensive rabies surveillance, prevention and control in Türkiye.

Keywords: Eastern and Southeastern Anatolia, G-L intergenic region, Rabies virus, N gene

Introduction

Rabies is a zoonotic infection that is transmitted from infected animals to humans through biting, contact of infected saliva with damaged skin or mucous membranes, and rarely, through high levels of aerosol exposure to the virus. Human-to-human transmission of rabies is an exceptional occurrence and has been reported only in cases of tissue and organ transplantation [1].

Rabies virus (RABV) belongs to the *Mononegavirales* order, *Rhabdoviridae* family, and *Lyssavirus* genus. Lyssaviruses are divided into seven genotypes and two phylogroups, comprising 17 species. The rabies virions are bullet-shaped, have a non-segmented, single-stranded negative RNA genome of about 12 kb, are enclosed within a helical nucleocapsid and surrounded by an envelope with glycoprotein spikes measuring 5-10 nm in size. Like all lyssaviruses, rabies virus contains five main genes, which are flanked by non-coding intergenic regions in a conserved order, namely 3'-N-P-M-G-L-5' [2]. The N gene, being highly conserved and easily detectable through RNA-based methods, is the most common target for diagnostic tests and phylogenetic typing. Among the non-coding intergenic regions, the G-L intergenic region is located between the G and L genes, which is highly susceptible to mutations. There is no immunological selection pressure on the G-L region, and the most variable region of the rabies virus genome. In general, studies of RABV phylogeny and dispersion have been performed through analysis of the complete G gene, G-L intergenic region, and/or partial or complete analysis of N gene [3-9].

For a long time in Türkiye, stray dogs have been considered the main reservoir of rabies virus [6,7]. It is known that interactions between stray animals and wild animals in the same areas create opportunities for transmission to different hosts [6,7]. In our country, rabies cases in red foxes *(Vulpes vulpes)* have been reported consistently, with occasional occurrences [7,8]. However, the Aegean region drew attention in 1996 when there was a small but continuous increase in rabies cases in foxes. Initially,

it was unclear whether this phenomenon occurring in wildlife was part of an endemic cycle in the wild [6]. Subsequently, it was reported that there was spillover from dogs to foxes, leading to the endemic establishment of fox rabies in Türkiye [7]. According to that situation, the oral vaccination campaign was initiated in 2008 in the western cities of Türkiye such as Manisa and İzmir [7]. The vaccination programmes currently continue for the prevention of rabies in both domestic animals and wildlife. Unfortunately, rabies, despite all efforts, remains endemic in Türkiye and poses a threat to public health.

It is known that molecular and phylogenetic analysis of RABV isolates from in field outbreaks are an important tool to determine the origin of the viruses and helps to predict its future occurrence cases and thus allows the adoption of prevention and control measures. The available information is relatively restricted to a few studies analyzing a limited number of viruses in Türkiye [6-8]. Moreover, very few of them belong to the viruses circulating in Eastern and Southeastern Anatolia regions sharing a land border with some countries where rabies is endemic.

This study presents the distribution of clades/subclades of rabies viruses from different animal species in the Eastern and Southeastern Anatolia regions of Türkiye between 2016 and 2021.

Material and Methods

Ethical Approval

This study was conducted with the permission of the Ministry of Agriculture and Forestry, General Directorate of Food and Control (permit number: E-71037622-806.01.03-1035577, dated 01.04.2021) and in accordance with the decisions of the Local Ethics Committee for Animal Experiments at Ankara University (AÜHADYEK; decision no. 2022-3-30, dated 02.02.2022).

Materials

The study utilized brain samples sent to the Rabies Diagnostic Laboratory of the Etlik Veterinary Control Center Research Institute, a national reference laboratory, from the Veterinary Control Institutes in the Eastern and Southeastern Anatolia regions between 2016 and 2021. Study materials (n=121) were selected from among the ones confirmed as positive for rabies virus according to their location (province), animal species and year.

RT-PCRs

RNA extracts, prepared using a commercial kit (MagNa Pure Compact Nucleic Acid Isolation Kit I; Roche, Germany), were subjected to RT-PCR using primers sets targeting the N gene and G-L intergenic region, as described elsewhere [3,9]. For the reverse transcription of viral RNA, cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RT-PCR was applied to obtain relevant products from the N gene and/or GL gene intergenic region of the rabies virus.

For the N gene region, the PCR protocol consisted of an initial denaturation at 95°C for 3 min, followed by a total of 35 cycles, each comprising denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. For the GL intergenic region, the PCR protocol included an initial denaturation at 94°C for 3 min, followed by a total of 35 cycles, with denaturation at 95°C for 45 sec, annealing at 51°C for 30 sec, extension at 72°C for 1 min and 30 sec, and a final extension at 72°C for 10 min.

After PCR, the obtained amplification products were visualized under UV light following agarose gel electrophoresis with 1% SafeView™ Classic (ABM, Canada). The same primers used for amplification were used for bidirectional sequencing of the obtained PCR products. Relevant sequences were obtained from the GenBank database for rabies viruses detected in Türkiye and other countries using the BLAST engine. The obtained sequences were aligned with the genomic sequences of reference viruses from GenBank and reported local viruses from other countries, using Aliview [10] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) software [11]. For phylogenetic analysis based on nucleotide sequences, the MEGA X program [12] was used. A Maximum-Likelihood method with the K2+I model was used to construct the phylogenetic tree for the N gene, and bootstrap analysis (1000 replicates) was performed. For the G-L intergenic region, a Maximum-Likelihood method with the K2+G model was used to construct the phylogenetic tree, and bootstrap analysis (1000 replicates) was performed.

The nucleotide(nt) identities were calculated by using the SIAS online tool *(http://imed.med.ucm.es/Tools/sias.html)*.

Results

Out of 121 samples tested RT-PCR, 88 from 21 different provinces (cities) produced the expected size amplicons, 1372 bp and 879 bp, of the N gene and/or G-L intergenic regions, respectively.

The phylogenetic analysis data reveal that rabies viruses circulating in domestic and/or wild animals in the Eastern and Southeastern Anatolia regions of Türkiye belong to three different subclades within the Cosmopolitan clade. These subclades are Middle East 1a (ME1a) (n=45), Middle East 2 (ME2) (n=20), and Central Asia 2 (CA2) (n=23). The distribution of rabies virus subclades

Table 1. *The distrubion of Rabies virus subclades identified according to the provinces*

City	Species (n)	Years					
		2016	2017	2018	2019	2020	2021
Adıyaman	Cattle (1)					ME1a	
	Dog (1)				ME1a		
Ağrı	Dog (3)			ME2	ME2		ME2
	Cattle (1)						ME1a
Ardahan	Dog (3)		ME2*	ME2			
	Wolf (1)		ME2				
Bayburt	Dog (1)			ME1a			
Bingöl	Cattle (2)						ME1a, CA2
	Dog (2)		ME1a		ME2		
	Wolf (1)			ME1a			
Bitlis	Cattle (2)						ME1a,CA2
	Dog (1)		ME1a				
Diyarbakır	Cattle (1)						CA2
	Dog (6)		CA2	ME1a	CA2		CA2**
	Jackal (1)			ME1a			
	Fox (1)		ME1a				
Elazığ	Cattle (1)						ME1a
	Dog (5)		ME1a*	ME1a	CA2		CA2
	Fox (1)		ME1a				
	Cat (1)		ME1a				
Erzincan	Dog (1)						ME1a
Erzurum	Dog (3)			ME1a			ME2, CA2
	Cat (1)			ME1a			
	Fox (1)			ME1a			
Gaziantep	Dog (3)					ME1a	ME1a, ME2
Iğdır	Dog (1)						ME2
	Cattle (1)				ME2		
Kahramanmaraş	Cattle (1)					ME1a	
	Goat (1)					ME1a	
	Dog (1)						ME2
	Fox (1)				ME1a		
Kars	Dog (3)			ME2	ME2		ME2
Malatya	Dog (5)	ME1a	ME1a	ME1a	ME1a		ME1a
	Fox (2)		ME1a			ME1a	
	Marten (1)				ME1a		
Mardin	Dog (3)		ME1a	ME1a			CA2
	Wolf (1)		ME1a				
Muş	Dog (2)			ME1a			ME2
Şanlıurfa	Dog (5)				CA2	CA2	CA2**
	Cattle (4)					ME1a	CA2, ME1a*
	Goat (1)					ME1a	
	Donkey (1)						CA2
	Horse (1)						ME1a
Tunceli	Sheep (1)				ME1a		
	Dog (1)			ME1a			
Van	Dog (6)			ME2	ME2	CA2	ME2, CA2*

** n=2 ** n=3*

Table 2. *The distrubition of Rabies virus subclades identified according to animal species (n)*

Species	Subclade (n)		Year					
			2016	2017	2018	2019	2020	2021
Dog (n=57)	ME2	18		2	4	4		8
	ME1a	21	1	6	8	2	1	3
	CA2	18		1		3	2	12
Cattle (n=13)	ME2	1				1		
	ME1a	8					2	6
	CA2	4					1	3
Fox (n=6)	ME1a	6		3	1	1	1	
Wolf (n=3)	ME2	1		1				
	ME1a	2		1	1			
Cat (n= 2)	ME1a	2		1	1			
Horse (n= 1)	ME1a	1						1
Donkey (n= 1)	CA2	1						1
Marten (n= 1)	ME1a	1				1		
Jackal (n= 1)	ME1a	1			1			
Sheep (n=1)	ME1a	1				1		
Goat (n=2)	ME1a	2					2	
Total		88	1	15	16	13	9	34

identified were shown in *Table 1* and *Table 2*, according to provinces and animal species, respectively. Additionally, the phylogenetic trees constructed using sequences of rabies viruses from selected animals representing different species, locations and years are shown in *Fig. 1* and *Fig. 2.*

In this part, the pairwise comparison of nucleotide sequences of our strains and strains deposited Genbank were examined in detail for every rabies subclades detected. The identities of the N gene nucleotide sequences of the rabies viruses obtained in this study with the reference strain NC_001542 were 87.46-92.96%. The local rabies viruses studied in this research shared 95.71-100% sequence identity to each other. Additionally, 93.27-100% and 81.34-100% nucleotide sequence identity to sequences previously reported from Türkiye and other countries sequences used in the tree *(Fig. 1)*, respectively. Our Turkish ME1a subclade and CA2 subclade rabies virus nucleotide sequences from shared 95.71-100% and 96.33-100% nucleotide identity among themselves. Also, nucleic acid sequences of the G-L intergenic region were aligned to obtain information on the genetic diversity of the RABVs detected in this study. Our strains shared 89.57-100% nt sequence identity with each other. In addition, they displayed 83.23-84.74% nt sequence identity with the reference strain NC_001542. They shared 88.99-99.22% nt and 71.42-99.42 nt sequence identity to the other RABVs previously identified in Türkiye and other countries used

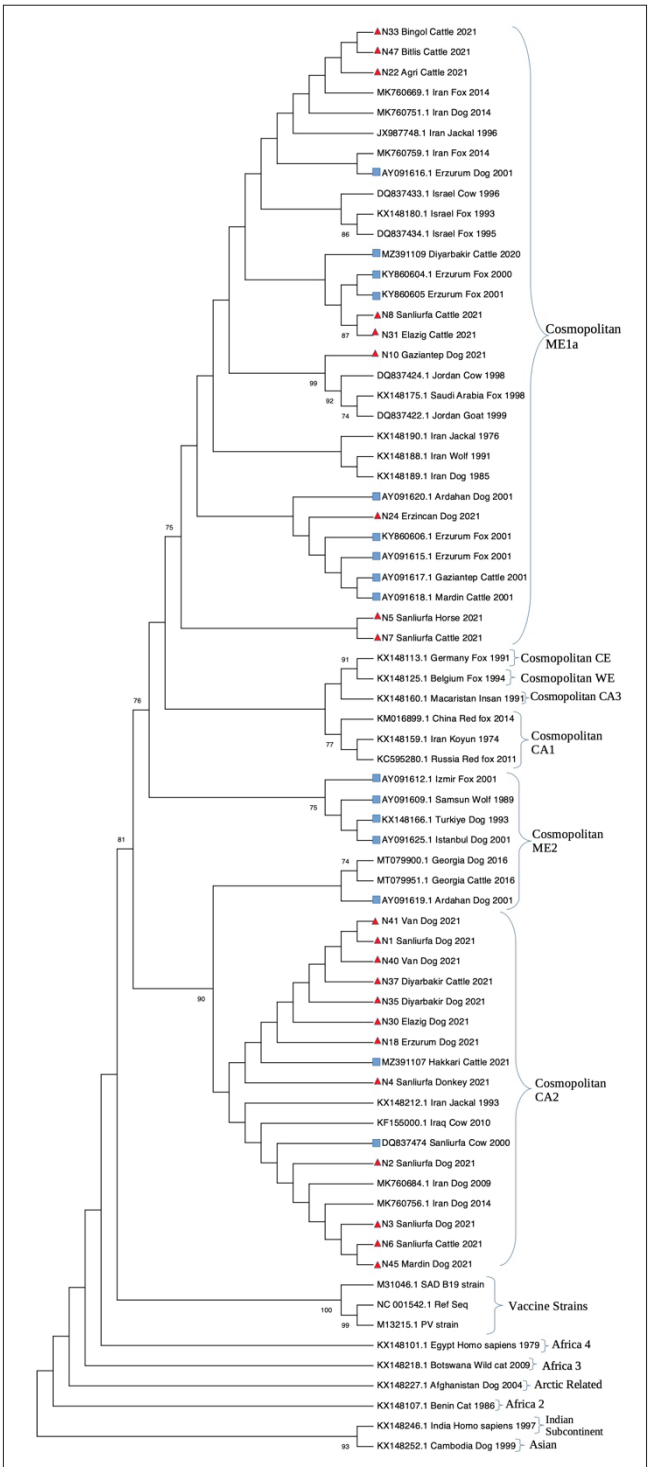

Fig 1. Phylogenetic tree based on the nucleotide (327 bp) of N gene of RABV. The phylogenetic trees were constructed using the Maximum Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Our strains and Turkish strains previously deposited in GenBank are indicated by red triangles and blue squares, respectively

in the tree *(Fig. 2)*, respectively. For the G-L gene region nucleotide identities among the RABVs identified as ME1a, ME2 and CA2 subclades in this study were 90.34-99.8%, 97.1-100% and 89.57-100%, respectively.

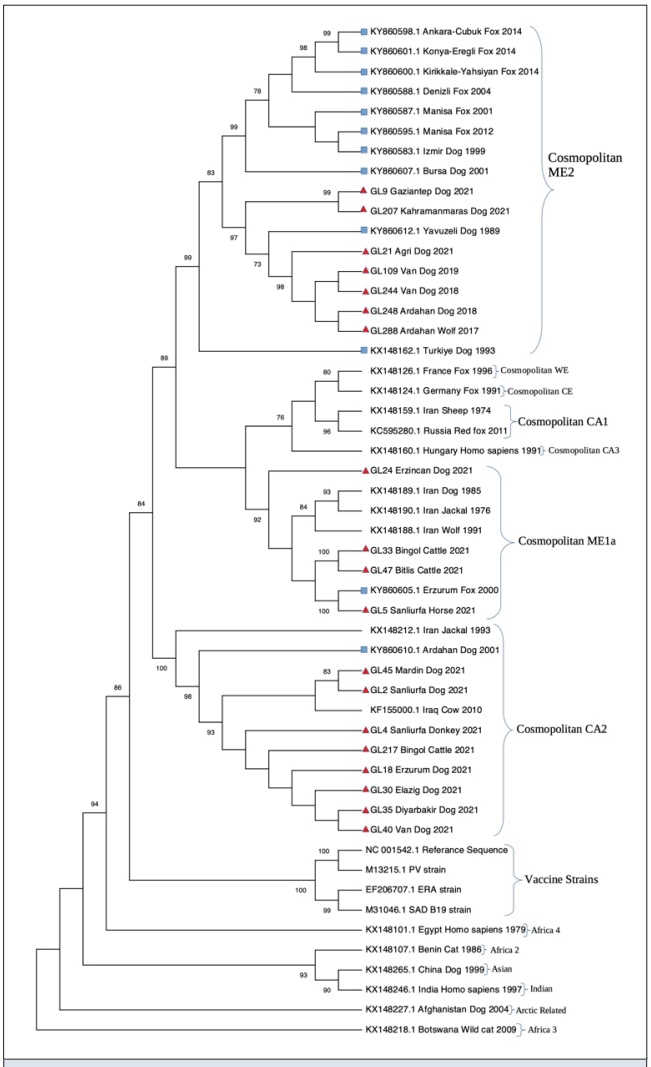

Fig 2. Phylogenetic tree based on the nucleotide (518 bp) of G-L intergenic region of RABV. The phylogenetic trees were constructed using the Maximum Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Our strains and Turkish strains previously deposited in GenBank are indicated by red triangles and blue squares, respectively

Discussion

Globally, rabies infection still causes the human deaths, with the majority being children. In developing countries today, dogs, including wildlife species, are considered to be primarily responsible for rabies cases and the spread of the disease. Currently, 99% of human deaths worldwide due to rabies are attributed to dog-mediated transmission [1].

In 2015, the World Health Organization (WHO), Food and Agriculture Organization (FAO), World Organization for Animal Health (WOAH), and the Global Alliance for Rabies Control (GARC) came together to prioritize the fight against rabies with a one health approach. The goal of this platform is to eliminate human deaths from dog-mediated rabies by the year 2030. Consequently, each country will plan its rabies control efforts considering its national and regional characteristics. Due to the limited availability of data, the inclusion of disease monitoring and surveillance has become a crucial component in the implementation of rabies programs [1].

Studies have revealed that there is a a strong correlation between genetic and geographical criteria, with rabies virus isolates forming genetic clusters based on the geographical regions [4-6]. In a previous study [6], Turkish rabies viruses, identified as Genotype 1 based on N gene sequences (327 bp) data, were classified in three different branches according to their geographical origins: the "Western Branch" from materials obtained from the western region of the country, the "Eastern Branch" and the "Northeast-Caucasian Branch" from different isolates from the eastern provinces and the Ardahan province, respectively. Currently, there is a limited number of sequences available for rabies viruses originating from Türkiye in the GenBank database [6-8]. For this reason, although full-length sequences of the N gene region were obtained, to supplement the data with relevant sequence information from previous studies conducted in the Eastern and Southeastern Anatolia regions, *Fig. 1* was prepared using partial sequences (327 bp) of the N gene region. The phylogenetic analysis of rabies virus from rabid animals in the Eastern and Southeastern Anatolia regions revealed the presence of three different clades *(Table 1, Fig. 1* and *Fig. 2)*, which can be evaluated based on the previously reported articles [6,13]. Specifically, the ME2 clade was associated with the Western branch, the ME1a clade with the Eastern branch, and the CA2 clade with the Northeastern-Caucasian branch. We believe that it will be more accurate to use subclade names in future research and epidemiological evaluations.

This study reports that rabies viruses identified in different species within the domestic and wildlife of the Eastern and Southeastern Anatolia regions exhibit distribution in 3 different subclades, district from other geographical regions as deposited GenBank and also a comprehensive project aiming at the molecular epidemiology of rabies virus in Türkiye, which we are currently carrying out (data not shown). This should raise a question about the geographic location of these regions where shares land borders with Georgia, Armenia, Azerbaijan to the northeast, Iran to the east, and Iraq and Syria to the southeast. Thus, rabies viruses identified in this study were clustered with those reported from neighboring countries in the phylogenetic trees *(Fig. 1, Fig. 2)* as described in previous studies [6,13]. As a matter of fact, the identities of N gene sequences between some of our isolates from dog and cattle sampled from different cities as Ağrı, Bingöl, Bitlis, Şanlıurfa and Mardin in 2021 and Iranian isolates (MK760669 from fox in 2014, MK760684 from dog in 2009 and MK760756 from dog in 2014) were 100%. Similarly, the identities of G-L gene regions sequences between some of our isolates

(from dog sampled from different cities as Mardin, Şanlıurfa, Erzurum, Elazığ, Diyarbakır, Van, and from a cattle from Bingöl in 2021) and Iraq isolates (KF155000; from a cattle sampled 2010) were 99.42%.

Another issue to be noted is the results of CA2 subclade of rabies virus. *Table 1* and *Table 2* shows that there are the increasing rates the CA2 subclade in dogs in the years 2019-2021, and, similarly, its detection in some domestic species (cattle and donkey) particularly in the year 2021. Although data from a limited number of materials could be used, it is our assessment that these results is related to the circulation of rabies virus in these regions, particularly through stray dogs.

In addition to canine-mediated rabies, the epidemiology of rabies in Türkiye is further complicated by the presence and geographical distribution of various reservoir species in wildlife that can transmit rabies. The data about wild animals from this study can be briefly summarized as follows. The viruses detected in wildlife animals (n=11) are all of the ME1a subclade, except for one (ME2) detected in a wolf in the year 2017. It is obviously that the limited number of viruses from animals in wildlife restricts the assessment of virus transmission between domestic and wildlife animals. However, it is possible that the prevalences of ME1a in samples from wildlife animals and dogs *(Table 2)*, particularly in 2017-2018, raises the question of the rabies virus cycle between wildlife and domestic carnivores, which warrants further investigation through advanced analyses.

It is generally accepted that the rabies can be controlled and eliminated by mass vaccination of reservoir animal populations. In Türkiye, the Ministry of Agriculture and Forestry initiated an oral vaccination campaign against rabies in wildlife in 2008. As a continuation of the oral vaccination campaigns in the Aegean region, 2008-2010, the "Rabies Disease Control Project in Türkiye," supported by the European Union, was implemented in 2014. The aerial vaccination was conducted once a year during the years 2014, 2015, and 2016. As a continuation of the mentioned project, aerial vaccination campaigns were conducted in the fall of 2019 [14]. Finally, in open sources, the statement reports that the rabies cases in the Eastern and Southeastern regions of Türkiye are originating from wildlife (foxes), and the oral vaccination is planned in the mentioned regions [15]. There is good synchronization between this statement and this study, which aims to report on subclades of rabies viruses in the geographical regions mentioned and raise some questions, for further studies, as: What species is the main factor of rabies transmission in the region? To what extent do stray dogs pose a threat to humans? What is the importance of stray dogs in the fight against rabies? It is highly likely that the rabies virus, which is transmitted from foxes to dogs, poses a significant threat to public health in the form of dog-associated rabies cases. Further studies on molecular epidemiology of rabies viruses from humans and, both wild and domestic animals, will contribute to the answering all these questions.

In conclusion, the oral vaccination campaigns conducted by the Ministry of Agriculture and Forestry, as well as the vaccination campaigns for owned/stray dogs and educational activities for the public, are of great importance in the context of controlling rabies. For the follow-up and success of vaccination studies, studies that present up-to-date data on the rates of rabies in different species and the molecular analysis of the circulating viruses etc. should be done continuously. We believe that people with knowledge about rabies, as well as official regulations, are an important component of the success of rabies eradication efforts in Türkiye.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D.A.Y.) upon reasonable request.

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Ethical Approval

This study was conducted with the permission of the Ministry of Agriculture and Forestry, General Directorate of Food and Control (permit number: E-71037622-806.01.03-1035577, dated 01.04.2021) and in accordance with the decisions of the Local Ethics Committee for Animal Experiments at Ankara University (AÜHADYEK; decision no. 2022-3-30, dated 02.02.2022).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DAY and FA conceived and planned the study design. DAY conducted the experiments and performed the molecular biology and bioinformatic analyses (alignments, phylogeny). DAY and FA interpreted the obtained data. DAY drafted and wrote the manuscript; FA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Case Report

Thermography Diagnosis of Medial Patellar Ligament Rupture in a Horse

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Abstract

In this report, thermography was evaluated as a portable diagnostic tool in addition to ultrasonography, which is frequently used in the diagnosis of medial patellar ligament tears in horses. Thermography revealed that the medial patellar ligament has difficulty absorbing quadriceps forces and that the cranial surface of the stifle joint shows elevated temperatures at rest. At the end of training, load sharing occurred between the cranial and caudal surfaces of the joint and the quadriceps muscle. Temperature changes captured by thermography may indicate the severity of the injury and help clinicians suspect ligament tears even without access to ultrasonography.

Keywords: Horse, Medial patellar ligament rupture, Thermography

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Introduction

Injury to the stifle joint [1], the largest and most complex joint in horses, is an essential cause of hindlimb lameness, especially in performance horses [2]. The location of lesions in the joint may vary according to the level of work, discipline, and age [3]. Patellar ligaments are potential causes of orthopedic problems. Lateral patellar ligament injuries are usually trauma-related and associated with an external wound [4]. However, more rare medial patellar ligament injuries may cause locking of the joint and permanent or temporary upward fixation of the patella. This condition may require medical or surgical treatment, depending on the severity of the lesion [5].

Although ligament laxity is often implicated as the cause of lameness, desmopathy of the patellar ligaments have begun to be described in horses. In studies showing the normal and abnormal structures of the patellar ligaments ultrasonographically, diseased conditions have been reported to vary between 4-18% [4].

Injuries to this joint are challenging to diagnose, the prevalence is unclear and poses a significant problem for clinicians [6]. When the cause of lameness is the stifle joint, radiography and ultrasonography are usually used to diagnose the lesions. Without calcification in tendon/ligament lesions, tissue details are not visible on radiographs due to low contrast [7]. Although ultrasonography is a proper diagnostic method for soft tissues in the stifle joint, not all structures may be fully defined due to the complex anatomy of the joint [8]. Although techniques such as computed tomography, magnetic resonance imaging, or arthroscopy provide valuable data as diagnostic methods in joint diseases, they have disadvantages, such as being expensive, requiring special equipment, and requiring general anesthesia [9,10]. Thermography, a diagnostic method in clinical practice, enables remote determination of physiological or pathological surface temperatures without the need for restraint of animals. In veterinary medicine, this technique has been used on farm [11] and companion animals [12] since the late 1950s until today. It has many advantages over other diagnostic methods, such as being non-contact, not requiring anesthesia, no need to hold the animal, providing real-time temperatures in superficial areas, simultaneous comparisons, and easy portability [11]. As mentioned, although each diagnostic method has different advantages, multiple methods are used to make a diagnosis due to the size and complexity of

the stifle joint in horses. Although previous studies have documented the use of the methods, we aimed to present ultrasonographic and thermographic data in a rare case of medial patellar ligament tear in horses, including measurement of surface temperatures. Thus, it would be valuable to include thermographic findings among the diagnostic methods to evaluate the stifle joint.

Case History

Ethical Approval

Informed consent was obtained from the animal owner to use the data obtained from the clinical examination.

Clinical Examination of the Horse

A presentation was made of an 11 years old German male jumping horse, weighing 650 kg, who was examined at the farm where he was found for constand cold lameness of the hind leg. We were informed that the horse has been training generally since the lameness started. The training program consisted of 20 min of warm-up and 20 min of galloping and jumping. Therefore, the horse presented was examined before the training, after 20 min of warm-up, and at the end of the training (after 40 min). Clinical examination, ultrasonographic, and thermographic examination were performed before training. Clinical examination and thermographic examination were again performed at 20 and 40 min. Radiographs of the stifle joint could not be taken because the X-ray equipment was not portable and could not be taken to the field.

It was learned that the horse had been lame for two months, and different medical treatments (triamcinolone acetonide ampoule, diclofenac sodium gel, and hyaluronic acid serum-intraarticular) were applied during this period. However, the horse's keeper also stated that no medication had been administered for the last two weeks. Physical examination revealed pain and tenderness in the left stifle joint. Inspection and palpation revealed no wound, crepitation, or joint swelling. Body temperature was within normal limits. During clinical examinations, it was observed that the horse's stride length was shortened at the trot, the haunch was kept at a low level, and the hoof tip rubbed the ground during stepping.

Thermographic Examination

Thermography measures the surface temperature of any object with a temperature above absolute zero [11], and as shown in *Fig 1*, hot spots are seen in red-white and cold spots in blue. Along with the initial clinical examination, thermographic images were taken at 20 and 40 min of training. Temperature changes were recorded on the left quadriceps muscle and cranial, lateral, and caudal surface of the left stifle joint *(Fig. 1)*. Three measurements were taken at each mentioned site and statistically analyzed.

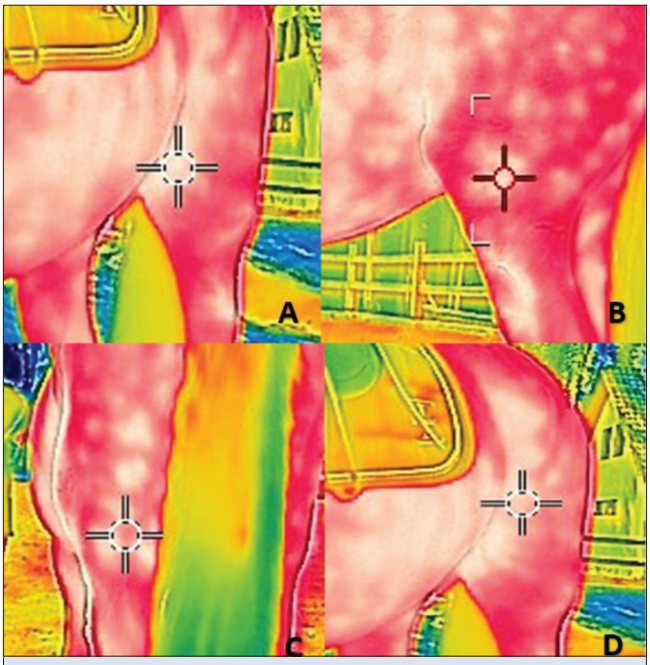

Fig 1. Surfaces from which thermographic images are taken. **A:** Cranial surface of the stifle joint, **B:** Lateral surface of the stifle joint, **C:** Caudal surface of the stifle joint, **D:** Quadriceps muscle

The emissivity value for subjects was 0.93, and all images were taken at the same distance (2 m) [13]. Temperature measurements from the regions shown in *Fig. 1* were analyzed with one-way ANOVA. The values were reported as means with standard deviation and subjected to analysis using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The statistical analyses were conducted at a significance level of $P<0.05$.

According to the results of the analysis, it was found that the temperature in the cranial surface of the stifle joint before training was significantly higher than in the other regions. When the anatomical regions were compared at 20 min of training, the highest temperature was found on the lateral surface of the stifle joint, followed by the cranial surface. The caudal surface of the stifle joint and the quadriceps muscle had statistically the lowest temperature, and these two regions were not different. At 40 min of training, the temperatures of the cranial surface of the stifle joint and quadriceps muscle were not significantly different. Still, they had significantly higher temperatures than the other two anatomical regions. Time-dependent changes in temperature measurements are shown in *Table 1*.

While the temperature changes from the anatomical regions according to time were in this way, statistical analysis was also performed according to the anatomical regions. Although the temperatures on the cranial surface of the stifle joint increased at 20 and 40 min, the difference between the pre-training and 20 min measurements was insignificant. However, there was a significant increase in the 40th min measurements compared to pre-training

Table 1. Temperature measurement values taken from anatomical regions according to time				
Anatomical Regions	**Before Training**	**20th Min. of Training**	**40th Min. of Training**	**P-Value**
Cranial Surface of Stifle	33.57±0.15^{b}	33.80±0.10^{b}	36.47±0.25^{a}	<0.001
The Lateral Surface of the Stifle	32.67±0.15^{b}	34.67±0.15^{a}	34.73±0.21^{a}	<0.001
The Caudal Surface of the Stifle	32.83±0.06^{c}	33.23±0.15^{b}	34.73±0.06^{a}	<0.001
Quadriceps Muscle	32.80±0.10^{c}	33.17±0.15^{b}	36.63±0.15^{a}	<0.001
Data presented mean±SD (n=3). Different superscripts show significantly differences (P<0.05)				

Table 2. Temperature measurement values according to anatomical regions					
Measurement Time	**Cranial Surface of Stifle**	**The Lateral Surface of the Stifle**	**The Caudal Surface of the Stifle**	**Quadriceps Muscle**	**P-Value**
Before training	33.57±0.15^{a}	32.67±0.15^{b}	32.83±0.06^{b}	32.80±0.10^{b}	<0.001
20th min. of training	33.80±0.10^{b}	34.67±0.15^{a}	33.23±0.15^{c}	33.17±0.15^{c}	<0.001
40th min. of tTraining	36.47±0.25^{a}	34.73±0.21^{b}	34.73±0.06^{b}	36.63±0.15^{a}	<0.001
Data presented mean±SD (n=3). Different superscripts show significantly differences (P<0.05)					

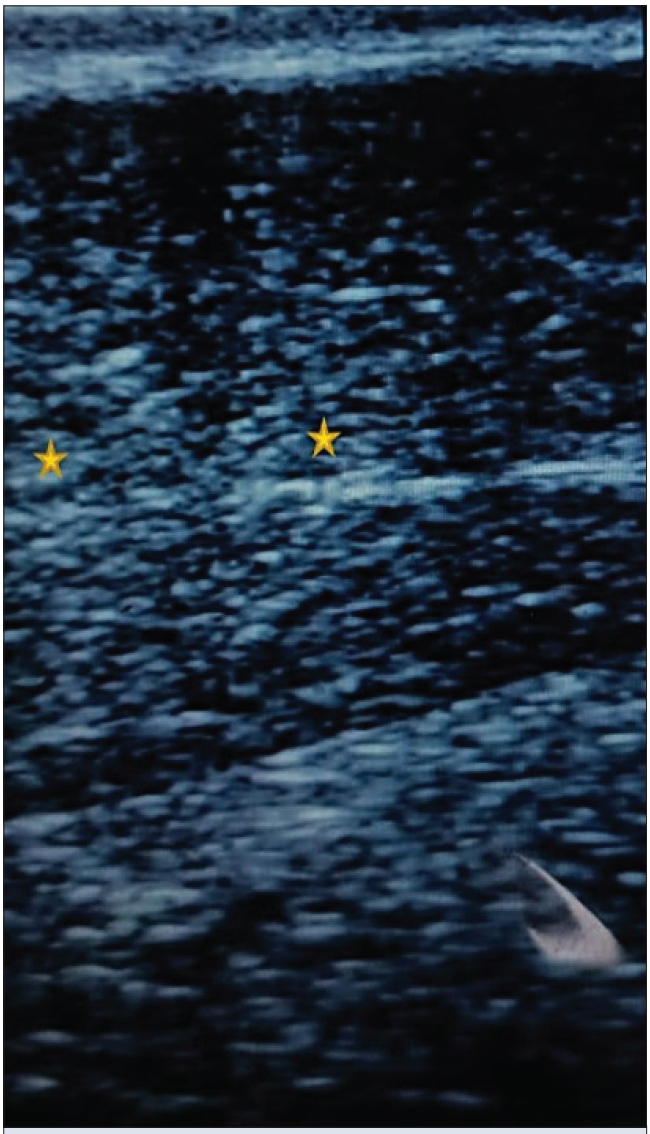

Fig 2. The yellow stars indicates the heterogeneous structure of the quadriceps muscle and echogenicity increases

and 20th min. Although there was no significant difference in the temperatures on the lateral surface of the stifle joint between the 20th and 40th min measurements, these measurements were significantly higher than the temperatures before training. In the analysis of temperature measurements taken from the caudal surface of the stifle joint and quadriceps muscle, it was determined that the temperatures gradually increased over time, which was significant *(Table 2)*.

Ultrasonographic Examination

The horse underwent an ultrasonographic examination of the stifle using an ultrasound machine (Mindray Digital Ultrasonic Diagnostic Imaging System DP-20 Vet, China) with a variable frequency (7-10 MHz) linear transducer. The horse was not sedated, and the hair was not clipped because he was being used for training. Machine parameters were adjusted as necessary to improve image quality. A stand-off pad was not used. Ultrasonography of the stifle joint was performed because pain and tenderness were detected in the left stifle joint on physical examination.

The bony structures, ligaments, and quadriceps muscle were evaluated in transverse and sagittal views of the left stifle joint in normal posture. By placing a probe cranio-proximal to the quadriceps muscles, images towards the tibia were controlled.

It was determined that the quadriceps muscle had a heterogeneous structure, and there were increases in echogenicity at some points *(Fig. 2)*.

It was noted that the medial and lateral trochleae were smooth. In the transverse plane, the intermediate patellar ligament was found to have an oval appearance and homogeneous echogenicity. The lateral patellar ligament

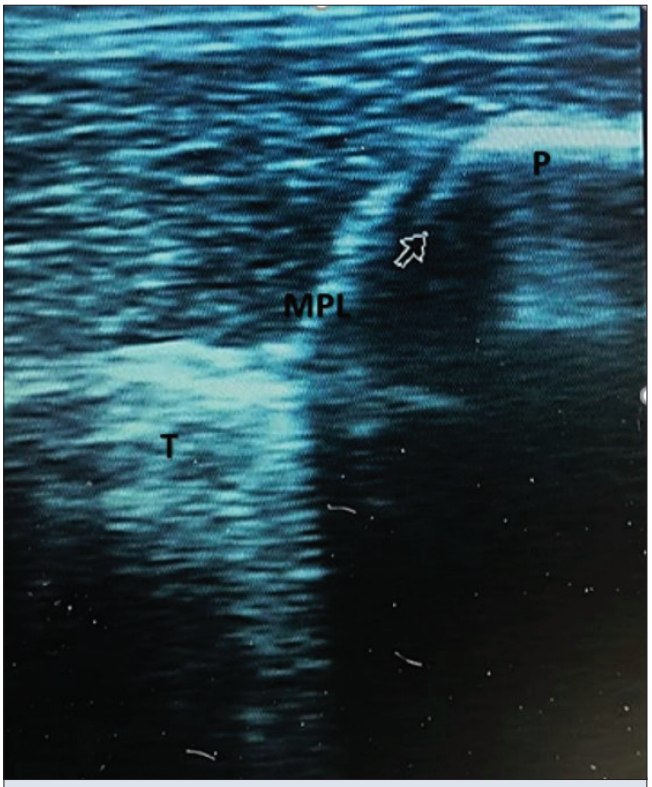

Fig 3. Ultrasonographic image of the medial patellar ligament. It is seen that the ligament is interrupted in the area indicated by the arrow (P: Patella, T: Tibia, MPL: Medial Patellar Ligament)

was evaluated by following the tibial tuberosity, but no irregular areas disrupting homogeneity were identified. In the examination of the medial patellar ligament, it was determined that the ligament was interrupted at the patella level in the transverse section, and an anechogenic area was formed in this area. And a ligament tear was diagnosed *(Fig. 3)*.

Discussion

Diseases related to the stifle joint are an important cause of hindlimb lameness in horses [1]. Although joint injuries are commonly reported in the literature, diagnosis is difficult for clinicians working in the field [6]. Although ultrasonography and radiography are often used for diagnosis, the disadvantage is that radiography lacks detail for lesions in soft tissues [7]. In addition, as in our case report, the fact that the radiography device is not portable also limits the clinicians in the field. Ultrasonography is used more in field studies because it can easily take images from soft tissues in different sections [8]. Although radiographs could not be obtained in the present case, ultrasonographic and thermographic images that can be used in field conditions were evaluated. The most important aim was identifying abnormalities in the lameness case clinically localized to the stifle joint. A diagnosis of medial patellar ligament tear was made with both ultrasound images and evaluation of superficial temperatures. In addition to being able to visualize the ligament tear, the temperature changes in this area also gave us important information. The horse's condition before training and the temperatures taken at 20 and 40 min into training were significantly different. At rest, the highest temperature was detected on the cranial surface of the stifle joint. Although the loads on joints and muscles have not been studied on live horses, a force of 8000 N was applied to analyze the load on the genu joint of a galloping horse in biomechanical studies. The strength of the patellar ligaments to withstand this load is 300 MPa. In the study that obtained these data, 1000 N proximal patellar tension was applied to represent the quadriceps force [14]. In the example study, it is understood how high the strength of the patellar structures and the quadriceps muscle must be to meet the load on the stifle joint. In the case we presented, we interpreted the high temperature of the cranial surface of the joint even in the resting state as the medial patellar ligament could not fully absorb the load and tried to counteract the quadriceps force coming from the dorsal side with the structures on the cranial surface of the joint. In the case we detected a medial patellar ligament tear by ultrasonography, we thought a ligament tear should be suspected by thermographic temperature measurement under field conditions. In this sense, thermographic data can provide essential data. We believe the joint and the surrounding structures were under more load because the patient was a jumping horse. In the meantime, the patient's anamnesis showed that he continued to train, so thermal images were taken 20 and 40 min into the training. Examination at 20 min revealed that the highest temperature was lateral to the stifle joint. The cranial surface ranked second, while the caudal surface and quadriceps muscle, which had the lowest temperature, did not differ. Several studies have suggested that the initiating factor of pathological processes is the inadequate ability of the tissues to cope with the mechanical load applied to the tendons [15,16]. This may be why the highest temperature before training is only on the cranial surface, whereas with training, the highest temperature is on the lateral surface. Because the load has gradually increased, other tissues may no longer be able to meet this load. This can explain the temperature rise. If the training were continued, we would encounter different findings thermographically. At 40 min, the highest temperature was again on the cranial surface of the joint, similar to the situation before training. However, this time, the temperature of the quadriceps muscle had also increased. Although these two anatomical regions had high temperatures, they were not statistically different. When we examined the change of the anatomical regions according to time, we found no difference between the temperature of the cranial surface of the joint before training and the temperature at 20 min. In comparison, there was a significant increase at

40 min. In the quadriceps muscle, we detected a steady increase in temperature over time. In the study by Frazer and colleagues [14], it was stated that the patellar structures, especially the quadriceps muscle, were under much load with the horse's galloping, as previously mentioned. After 20 min of training, he galloped and jumped obstacles in his daily training until 40 min were completed. In parallel with the literature, we think that the cranial surface of the joint and the quadriceps muscle temperature increased as the medial patellar ligament could not meet the load with more load on the leg. We found that although the temperature on the lateral surface of the joint increased in the 20th and 40th min compared to the pre-training period, there was no difference between them.

In conclusion, our case report provides valuable data for the diagnosis of medial patellar tear and the temperature of the articular surfaces and quadriceps muscle. When all our data are evaluated, we think that in medial patellar ligament tears, the temperature increases only on the cranial surface of the stifle joint at rest due to increased load; after 20 min of warm-up, the cranial surface and lateral surface are also under load, and when the training is terminated, this load is shared between the cranial, caudal surface of the joint and the quadriceps muscles. We can say that the lateral surface of the joint is under less load after galloping and jumping hurdles compared to other anatomical regions. The temperature differences identified in the case report, combined with clinical and ultrasonographic examination, indicated a pathology. Veterinarians working in the field should also evaluate superficial temperatures in this way. More cases are needed to confirm these results. In addition, we would like to emphasize that ultrasonography is very useful for diagnosis without portable X-rays for clinicians working in the field. In addition, a ligament tear can be suspected by determining temperature increases by thermographic examination. Thermography can provide significant findings as a diagnostic method.

Availability of Data and Materials

The data that support the findings of this case report are available from the corresponding author (E. Dogan) upon reasonable request.

Acknowledgments

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Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

Clinical, ultrasonographic and thermographic examination was done by ED, OD and ABD. ED analyzed and interpreted the data. ED, OD, ABD wrote the paper. Authors submitted the article together.

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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf) and "COPE Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/u2/Best_Practice.pdf).

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

• Privacy

The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

• Authorship and Authors Rights

The authors of the manuscripts sent to Kafkas Universitesi Veteriner Fakultesi Dergisi must have contributed significantly to the design, execution or interpretation of the study. For example, in view of the research and publication ethics as well as authors rights, it is not acceptable to include those as authors who do not actively contribute to the research but just only help in writing or data collection processes, which may not require any scientific knowledge. All the authors in a publication should be in agreement of the names and the orders of the authors in the manuscript.

The competence of the authors to the subject of the study is evaluated by the editor within the framework of deontological rules and the professional fields of each author.

The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

• Originality of Research Findings

The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

• Similarity

Articles submitted to the journal are subjected to similarity analysis using appropriate software (iThenticate by CrossCheck) at the beginning and at every required stage. If unethical similarities are detected regardless of the rate of similarity, this situation is reported to the authors and corrections are requested or articles containing excessive similarities are rejected at the first evaluation stage without being evaluated.

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Kafkas Universitesi Veteriner Fakültesi Dergisi applies publication ethics and verifies the originality of content submitted before publication and checks all submitted manuscripts for plagiarism/self-plagiarism, similarity and duplication. All submitted manuscripts are meticilously screened by a similarity detection software (iThenticate by CrossCheck). Papers previously presented at scientific meetings and published only as an "abstract" should be indicated in the Title Page file as stated in the "Guidance for Authors". Authors do not have the right to use entire paragraphs from their previous publications into a new submission. These actions are also considered as a plagiarism. In any case, the manuscript should be original in terms of scientific contents and writing. In the event of alleged or suspected research misconduct, the Editorial Board will follow and act in accordance with "COPE Guidelines".

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Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

Authors may think that their work should be published in multi parts that complement each other. For this, each part of the article should be titled "Part-I", "Part-II" and submitted to the journal "simultaneously". This issue can be evaluated by the editor-in-chief/subject editors/ referees who may suggest that the article can be published in parts or as a whole. In addition, rejection of a submission presented in parts means that all parts will be rejected.

• Animal Rights and Ethics

The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

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The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

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In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/ subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

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After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

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If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

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Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

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Kafkas Universitesi Veteriner Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veteriner Fakultesi Dergisi are available online, free of charge at https://vetdergikafkas.org/archive.php.

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• **Preliminary Evaluation Process**

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• **Peer-review Process**

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• **Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

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The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

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- Competing Interests

- Authors' Contributions

Further considerations

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• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided