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Review

A Systematic Review of IoT Technology and Applications in Animals

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Abstract

Precision Livestock Farming (PLF) is a mechanism that manages a production system. This mechanism includes mathematical models and controllable inputs that can predict inputs with processes and results that can be monitored periodically. These parameters of PLF systems can improve resource use efficiency and reduce cultivation costs. Many situations, such as the behaviour of animals on farms, their nutrition, estrus cycles, and epidemics, can be monitored with wearable devices containing various sensors. However, real-time monitoring of the data collected by these devices is possible with Internet of Things (IoT) technology. IoT is a multi-layered network that enables sensors within the system to communicate with each other and implement certain decisions when necessary. Sensors and IoT devices extract information from the raw data they collect from the environment, which is then shared with other objects, devices, or servers via the internet. The real-time data collection, processing, and analysis provided by IoT enables improvements in the management of animal farms. This systematic review addresses IoT concepts and applications in the livestock sector from a systematic perspective for different animal farms.

Keywords: Animal husbandry, Aquaculture, Beekeeping, IoT, Precision livestock farming

Introduction

Livestock farming and agriculture play a key role in the economies of many countries. Livestock farming is an agricultural and economic activity in which various animal species are raised, generally for the benefit of people, to obtain products such as meat, milk, eggs, and wool or to sell the animals. It can be carried out to supply animal products for animal husbandry, food production, textiles, industry, and other industries. This activity often includes nutrition, shelter, health care, reproduction, and other management practices. Increasing urbanisation limits the amount of land available for animal farms; However, the demand for animal products is increasing day by day. Due to limited land and other limited natural resources, farmers are forced to feed more animals in smaller areas [1].

Feed and disease are among the most basic cost elements in livestock farming. Proper nutrition of animals is important for both the health of the animal itself and the efficiency of the products obtained from the animal [2]. Since animals live together in shelters, if one of them contracts an infectious disease, it may result in the loss of many animals. Therefore, to reduce animal losses in animal husbandry and increase animal product yield, the nutritional and health status of animals must be regularly monitored. In traditional animal husbandry, all of these are carried out by farm workers. Constant monitoring is required to prevent problems. However, since decisions are made by people, oversights or disruptions may occur due to humanitarian reasons in this process. Employing more personnel to ensure adequate monitoring brings additional costs.

As in many fields, the use of information technologies in the livestock sector is becoming more widespread day by day. In particular, internet of things applications have made rapid progress in smart livestock solutions. A sensor is a device that can measure and detect a physical, chemical, or biological condition. Smart livestock farming is a system that enables the health and welfare of animals to be monitored and analysed using various sensors

integrated into the animals and/or the environment in which the animals live and to send feedback to the farmer and/or veterinarian in case of a critical situation [3].

The IoT is a technology that connects various objects without the need for human support. It has a multi-layered structure that includes both hardware and software. IoT systems include sensors and actuators. Sensors collect data from the environment, and actuators create commands based on the data and transform them into physical actions [4]. Collected data is usually stored in cloud-based areas, providing real-time monitoring. The stored data is used to detect deviations from normal or abnormal conditions. In this detection phase, artificial intelligence (AI) or machine learning (ML) algorithms are generally used. Sensors are usually integrated into a wearable device and placed on the animal [5]. When a situation that requires intervention is detected as a result of the analysis, techniques such as Radio Frequency Identification (RFID) and QR codes are used to enable IoT devices to identify the animal [6]. In an IoT system, human effort to control, protect, maintain, and monitor these smart devices should be minimal. With the correct interpretation of data from sensors, cost loss due to animal diseases can be reduced, and animal life cycles can be improved.

This study aimed to systematically review experimental studies analysing IoT systems in the field of animal husbandry. In this comprehensive review, state-of-the-art IoT systems developed for livestock and poultry, fish farms, and beekeeping are thoroughly examined and comparatively presented.

Material and Methods

The general framework for the use of IoT technology in animals is presented in *Fig. 1*. As seen in this flow chart, data collected from animals with sensors is transferred to devices such as computers, tablets, and phones. After the data is analysed by farmers, veterinarians, or experts, animals are intervened when necessary.

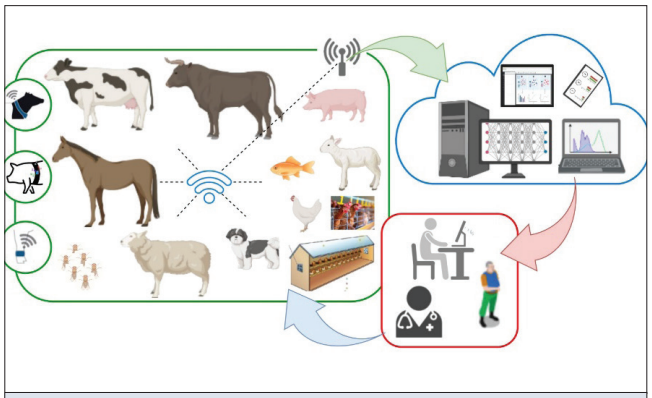

Fig 1. The general framework for IoT technology in livestock farming

In this review, studies on IoT-based livestock systems for different animal species were examined. The studies presented are limited to the years 2013-2023 to include current technology. Conference publications were not included, and only articles were focused on. Studies whose full text was not published or published in a language other than English were not included in this review. Since the focus of this review is IoT, studies conducted solely with information technologies such as image/audio processing have also been ignored. The research was conducted through Google Scholar, and the keywords used are as follows: IoT for animal estrus/calving/fertility, IoT for animal lameness, IoT for animal breeding, IoT for animal feeding, IoT for animal health, IoT for animal behaviour, IoT for fish farming, IoT for animal location tracking, IoT for bee/hive, IoT for poultry/chicken.

The results of the literature review are presented under the titles of animal health monitoring, animal activity detection, animal location tracking, animal smart feeding, animal estrus/calving detection, animal lameness detection, IoT for fish farming, IoT for beekeeping, and IoT for poultry management. Some studies may contain more than one title. For example, in some estrus/calving/lameness detection studies, the activities of the animals are also detected. Studies on animal health monitoring and animal smart feeding may also include animal location tracking. This systematic review, it was examined 23 studies for animal health monitoring, 6 studies for animal behaviour detection, 11 studies for estrus/calving detection, 7 studies for animal location tracking, 6 studies for animal smart feeding, 2 studies for lameness detection, 5 studies for beekeeping, 10 studies for fish farming, 6 studies for smart poultry. As a result, a total of 76 articles were reviewed.

Internet of Things (IoT)

IoT is a technology that provides the ability to exchange data in real time between multiple different smart devices [7]. In this way, data is collected by communicating between various devices, and this data is transmitted to a cloud system or an end device through a gateway using communication protocols such as Wi-Fi and Bluetooth [8]. There are 5 basic components in the IoT system: smart devices, gateway, data storage unit, IoT application, and graphical user interface. Smart devices consist of devices that can exchange data, such as sensors. Gateway allows data to be transmitted to other devices and includes encryption methods for data security by regulating the state of the network. Data storage is used to collect incoming data. Data coming from smart devices at various periods must be stored somewhere to be monitored and analysed. This data can be stored on a server or in a cloud system. IoT applications are software that integrates data from smart devices. It may include technologies such as

machine learning and artificial intelligence to analyse data and make various decisions based on this data. The decisions taken are transmitted to the IoT device, allowing it to respond according to the incoming input. Graphical user interfaces are interfaces, such as a mobile application or website, used to monitor and control incoming data [9].

The Internet of Things is a network of subnets in which objects, animals, or people are equipped with unique identifiers and can enable data transfer without requiring human-to-human or human-computer interaction [10]. IoT architecture for livestock farming generally consists of four layers, and these layers are given in *Fig. 2* [11,12].

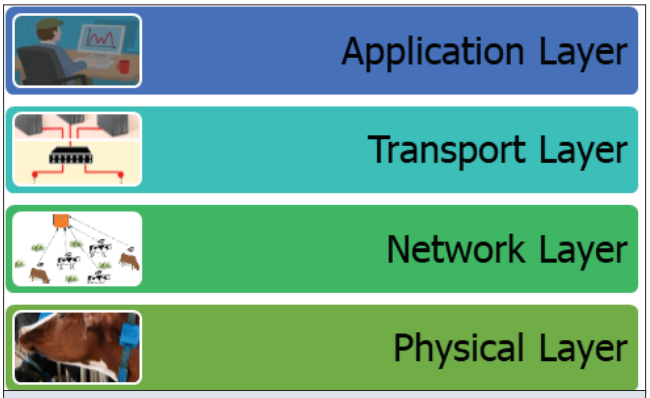

Fig 2. IoT livestock network architecture

Application layer: It is the top layer of the IoT network architecture and provides communication and interaction between IoT devices and applications [11]. The application layer is the most visible and user-friendly part of the IoT system. IoT applications in livestock farming are generally used for purposes such as tracking animals, monitoring health status, and farm management. The application layer supports various protocols and functions for such applications. In an IoT-based livestock application, the application layer supports functions such as data collection, data analysis, decision support, and application interface [13]. At the application layer, several different protocols can be used to enable data communication and interaction in IoT systems. Hypertext Transfer Protocol (HTTP), Message Queuing Telemetry Transport (MQTT), Constrained Application Protocol (CoAP), Advanced Message Queuing Protocol (AMQP), WebSocket, and Data Distribution Service (DDS) protocols are commonly used in IoT-based livestock applications [14,15].

Transport Layer: Acts as a link between the application layer and the network layer to enable communication between hardware and software. This layer allows animal information to be collected, transmitted, and analysed through sensors. It also ensures secure and error-free transmission of the obtained information using transmission control protocol (TCP) [12]. In this way, the health status of the animals, their movements, and other important information are reliably transmitted and analysed.

Network Layer: Transmits data as digital signals over the network to relevant platforms. This layer may contain only one relay point, with one interface connected to the sensor network and the other interface connected to the Internet [11]. It is also the base layer that monitors livestock applications such as animal health monitoring, tracking pet observation, etc., and transmits the information to the application layer. According to the concept of IoT network architecture, all wearable devices and sensors in livestock use 6LoWPAN and IPv6 systems to transmit data via the IEEE 802.15.4 protocol [12,16].

Physical layer: It is the lowest layer in the IoT network architecture and undertakes the task of collecting data through RFID, sensors, etc., and converting it into readable digital signals [11]. This layer monitors and measures several parameters related to the health status of the animals, such as their movements, body temperature, and digestive activity. It uses sensors and actuators to continuously monitor relevant parameters. Sensors collect data about the animal or the environment and convert it into analogue or digital signals. Z-wave, EPC-Global, LTE, and IEEE 802.15.4 are standards of the physical layer. The most popular among these is IEEE 802.15.4. Because IEEE 802.15.4 is less complex than others and has lower power consumption and cost [12].

Sensor Technologies

Sensors are devices that process physical information and convert it into electronic signals. IoT applications detect data from the outside world and convert them into digital and usable signals, enabling them to collect data from the outside world for functions such as actions and decisions. These data, obtained by measuring a physical, chemical, or biological condition or phenomenon, are used to predict deviations or abnormalities. In IoT applications, various sensors can communicate by sending and receiving data to each other over the internet. In IoT-based Precision Livestock Management applications, sensors are used to measure the suitability of the environment where animals live and to collect data about the animal's physical condition. This collected data is processed and analysed to monitor or detect various situations in advance. Sensor technologies and their purposes of usage in Precision Livestock Management applications are given in *Table I*.

Sensors can be grouped into external and invasive sensors. External sensors are usually mounted on a wearable device and placed on the animal's body. In this context, a wide variety of sensors are used. Accelerometers and pedometers are widely used to monitor animals' activity. Increased mobility in animals may be a sign of an estrus

period, and a decrease may be a sign of lameness [17,18]. The health of the animals and the quality of the environment can be monitored with temperature and humidity sensors. Turning on a fan or heater depending Riaboff on the increase or decrease in temperature provides an increasing effect on animal welfare. It is important to monitor as increases in humidity levels can cause stress in animals. Increased humidity and temperature will also create a suitable environment for the transmission of diseases. Low humidity can cause breathing problems and result in dehydration. Animal sounds can be used for situations such as disease and estrus monitoring [19-21]. GPS can be used for activity tracking in conjunction with activity sensors, as well as tracking the location of animals. In addition to sensors, images collected from cameras and sound data collected from microphones are used to track information such as the activity of animals and disease status.

Invasive sensors are often used to monitor physiological measurements such as core body temperature. It is administered either by the animal swallowing it or by implanting it into the animal. Invasive sensors can directly measure factors for monitoring animal health [22]. Therefore, they produce more accurate data than external sensors. They also enable the monitoring of parameters such as hormones, which are not possible to obtain with external sensors. However, since external sensors are integrated into animals with wearable devices, they are both reusable and easier to apply, causing less stress in animals. Also, it is lower in cost.

Sensors for animals are placed with wearable devices such as neck collars, headphones, and ankle collars that are attached directly to the animals. The data to be collected is the main element that determines where the device should be placed on the animal [23]. These sensors usually need to be in contact with the body, but hair on the animals can make direct measurement difficult. Additionally, wearable sensors mustn't be uncomfortable or heavy for animals; otherwise, the animal will try to get rid of the sensor. Considering the environment and activities of the animals, they need to be water and impact-resistant and long-lasting. Another important element for sensors is that they require energy to collect and transmit data. This energy is provided by batteries. Since frequent battery replacement is a difficult process for animals, it is also important that the energy source to be used is efficient [24].

Cloud Computing

The rapid development in technology has led to an increase in device usage and the number of users. Thus, data accumulation also increased. Users and companies want to store this accumulated data, access it whenever they want, and perform various analyses. However, the increase in data to be stored has also increased storage costs. Cloud computing is the general name for internet-based computing services. It offers an infrastructure where data can be accessed and shared via devices connected to the internet, such as computers and tablets. In addition to storing data, cloud computing also provides a platform where data can be analysed with artificial intelligence and machine learning [25].

Cloud computing services meet the need to store and analyze data in many IoT systems. In smart animal husbandry, data needs to be stored to monitor the welfare of the animals. Additionally, the detection of unusual situations can be made based on the analysis of historical data. In IoT-based smart livestock systems, developers use cloud computing to store data and/or detect abnormalities with machine learning algorithms using stored historical data [6].

Machine Learning

Machine learning is a subfield of artificial intelligence. The model obtained from data trained with algorithms is used to predict similar situations. The algorithm's ability to learn accurately depends on the good representation and size of the data [26].

There are two basic learning approaches in machine learning: supervised and unsupervised. Supervised learning enables a model to make specific inferences from input data or create a mapping corresponding to a specific output. For the algorithm to learn a specific input-output relationship, it must be trained with a labelled dataset [27]. Classification and regression problems are within the scope of supervised learning. Unsupervised learning is used to discover natural groupings or relationships between features in a data set, but data samples are not given labels. Using the similarity measures, it determines which group the unknown data may belong to based on its proximity to other data points [28]. Clustering is a common application area studied under unsupervised learning. Deep learning is an algorithm that can learn from large amounts of data using artificial neural networks that model the way the human brain works. It contains at least one multilayer artificial neural network and is capable of obtaining more precise results than traditional machine learning methods when a sufficient amount of data is available [29].

In IoT-based smart livestock systems, the actuator is responsible for alerting the farmer and/or veterinarians or automatically taking some measures within the system if a certain critical situation occurs. Nowadays, it is quite common to use predefined threshold values to determine the critical state. For example, they are sending a notification to the farmer or turning on a fan if the animal's body temperature is above a certain threshold. In

Table 1. Sensor technologies and their purposes of usage		
Sensor	**Function**	**Application**
Temperature sensor	Determine the temperature of an animal or environment	Animal health tracking, environment temperature monitoring
Humidity sensor	Determine the humidity level of an animal body or environment	Animal health tracking, environment humidity monitoring
Microphone	Use to collect sounds of animals	Animal health tracking, estrus detection, swarm bee, flock poultry farming
Camera	Use to monitor the environment and animals	Animal activity, estrus, lameness detection
GPS	A satellite system used to determine the location on Earth	Animal location tracking, activity detection, smart feeding
Accelerometer	Use to detect and track activities of animals	Motion, lameness, mastitis, estrus, feeding, rumination, position detection
Heart rate/pulsometer	Use to measure how many times the heart beats per minute	Heart rate
Respiration	To determine respiratory rate and pattern by monitoring breathing	Animal health tracking
Pedometer	To count the steps taken by animals in a certain period	Activity tracking, estrus/calving/lameness detection
Ruminal sensors	Use to monitor and evaluate certain parameters in the digestive system of animals	Meal tracking, chewing, digestion, and nutrition according to pH in the rumen system
Rumination sensor	Use to track chewing and digestion processes in the digestive system	Animal health tracking, feeding
Saliva sensor	Use to measure specific components in saliva, especially biochemical or biological parameters	Animal health, hormone, and nutrition monitoring
Gas sensor	Detect level of different gases (like CO_2, NH_3, CH_3 etc.) in the environment	Monitoring the air quality in the environments where animals live
Load sensor	Use to measure the weight of the animal	Animal health tracking, smart feeding
Luminance	Use to measure environmental light levels to adjust the light level	Animal health and environment tracking
Posture sensor	Used to monitor and evaluate body position and physical activities	Animal health tracking, Animal activity recognition
Biosensors	Use to detect a biological reaction by placing it inside the bodies of animals and measure the information obtained from this reaction	Feeding, estrus, disease
RFID	Use to identify and track animals using radio frequency	Animal location tracking, animal identification
Ultrasonic sensor	Use ultrasonic sound waves to determine the location of an animal, silo and calculate its distance from the location	Smart feeding, fish farming

some systems, predictions of machine learning algorithms are used instead of threshold values [29]. These algorithms are used to make real-time predictions about the condition of animals or the environment with data from sensors and cameras. In this way, when critical situations arise, automatic solutions can be provided through actuators, or notifications can be sent to authorised persons.

Communication Protocols

Connectivity is the basic need of an IoT system. Devices within the IoT system must be connected with a communication protocol to exchange data. *Table 2* shows the advantages and disadvantages of various communication protocols compared to each other. Animal farms are generally established in areas far from the city centre. This may cause an interruption to the internet, which is one of the basic building blocks of IoT. It is important to determine the correct communication protocol for the healthy functioning of the system. The correct protocol depends on the system's requirements. The area covered by the animal farm where the IoT system will be installed determines how far the IoT devices need to communicate. The power consumed for data transmission is another decisive factor. Because it is preferred that batteries are not changed frequently in the field, another important factor is that the costs of different protocols are different.

LoRa is generally preferred for data transmission over longer distances. SigFox is widely used, like LoRa, because it can provide low-power real-time monitoring on large farms. ZigBee is preferred in shorter-range applications such as indoor monitoring. Similarly, Wi-Fi and Bluetooth

Table 2. *Communication protocols and their technical specifications*

Protocol	Frequency Band	Transmission Range	Data Rate
Bluetooth	2.4 GHz	Indoor: Up to 10 m, Outdoor: Up to 1 km	1-24 Mbps
IEEE 802.11	2.4 GHz	Indoor: 20-70 m, Outdoor: 100-250 m	11-54 Mbps
ZigBee	2.4 GHz	10-100 m	Up to 250 Kbps
Wi-Fi	2.4-5 GHz	15-45 m	54-450 Mbps
LoRa	470-928 MHz, country-specific	Urban: 2-5 km, Rural: 5-15 km	0.3-50 Kbps
IEEE 802.15.4	868/915/2400 MHz	10 m	200-250 Kbps
GPRS/3G/4G/5G	850, 900, 1800, 1900 MHz, 3.3-3.8 GHz	Cellular area	7-300 Mbps
XBee	2.4 GHz/902-928 MHz /865-868 MHz	2 km	250 Kbps, 1 Mbps
MQTT	-	-	Up to 256 Mbps
SigFox	900 MHz	Rural: 30-50 km, Urban: 3-10 km	10-1000 bps
RF	<1 GHz	-	-
RFID	125KHz-915 MHz	3 m	400 Kbps

are also commonly used to monitor animals on a small scale or in confined spaces. It provides a high sampling rate and a high volume of data transmission. Another advantage of Wi-Fi is that it is easy to implement [30].

Results

Smart Agriculture and animal husbandry is an approach to animal husbandry that includes the use of modern technologies instead of traditional methods. This approach involves the use of innovative technologies such as sensors, data analytics, artificial intelligence, and internet connectivity. Smart livestock applications aim to make livestock activities more efficient, sustainable, and profitable. Thanks to these technologies, farmers can remotely monitor their fields, animals, and environmental conditions, increase productivity, and ensure more efficient use of resources. Smart farming has significant potential for the future of livestock farming and represents the digital transformation of the livestock industry. The taxonomy of smart animal husbandry applications is given in *Fig. 3*.

Today, smart agriculture is used to address many problems. In this review study, IoT applications were examined under the headings of precision livestock farming, health monitoring, tracking animal location, smart feeding, estrus/calving detection, lameness detection, animal activity detection, fish farming, precision beekeeping, and poultry.

Precision Livestock Farming

Livestock farming is a type of animal husbandry carried out for the production of foods that have an important place in human nutrition, such as meat and milk. In addition to animals raised for meat and milk, such as cows, cattle, buffalo, sheep, and goats, animals such as chickens and turkeys raised for meat and eggs are also included in the scope of livestock. Suitable land is needed for animals to shelter and continue their natural lives, and shelters such as barns and coops are needed for feeding animals. In addition to obtaining meat and eggs directly from livestock, by-products such as butter, cream, and cheese are also obtained. To increase the quantity and quality of the product obtained, animals of good breeds should be preferred, and animals should be fed with quality feed. Although efforts are made to increase productivity with the use of organic feed in traditional livestock farming, it includes various difficulties because it is based on constant monitoring and control [31].

Precision livestock farming (PLF) is the continuous monitoring of factors that will directly increase product yields, such as the location, health status, nutrition, and reproduction of animals, with the help of digital systems. These systems, also called livestock monitoring and control, are provided with technical devices such as cameras, GPS, various sensors, and RFID placed on livestock land and animals. Thus, information such as

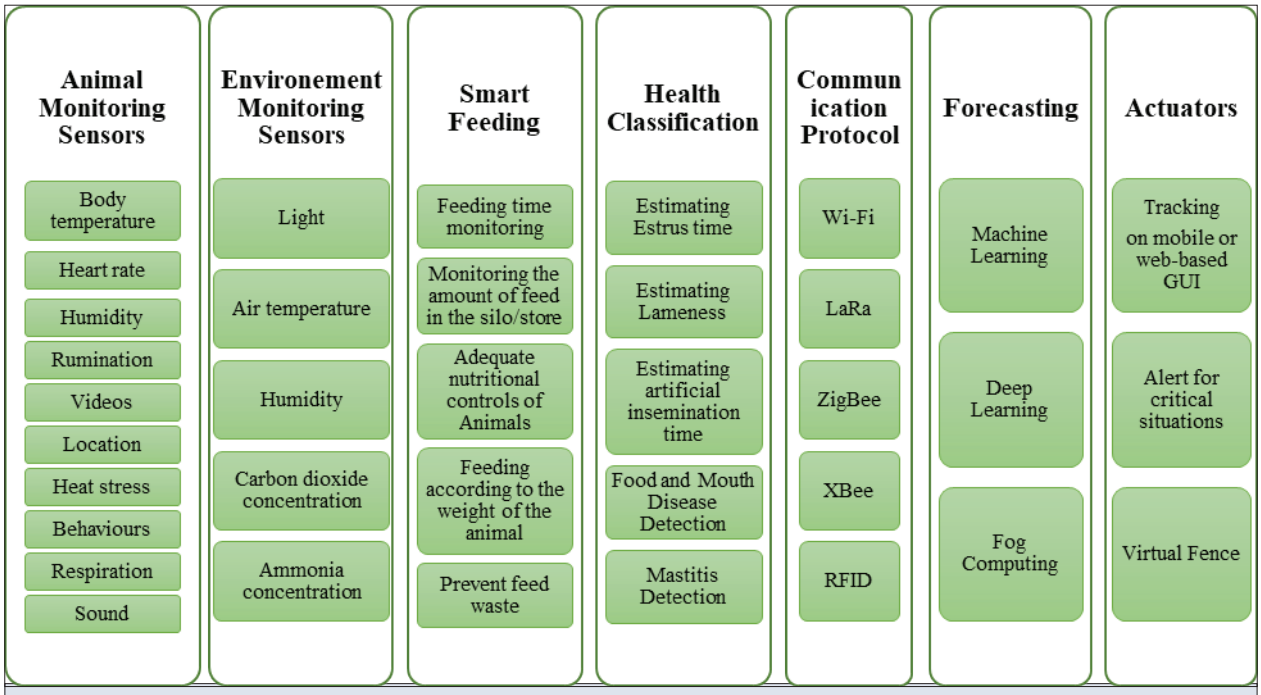

Fig 3. Taxonomy of applications in smart farming

the health status of the animals, their nutritional levels, and their locations can be monitored regularly [32]. Air and climate control in these living spaces is provided by sensors placed in livestock areas and/or indoor living spaces of animals. Data collected by sensor networks can be visualised and monitored through mobile or web-based applications, and the responsible person can be informed in critical situations [33]. PLF systems include modules such as livestock health monitoring, tracking their location, and smart feeding [32]. Within the scope of the study, each submodule was examined under a separate heading.

Health Monitoring

In traditional livestock farming, the officer checks the health status of animals manually. If a disease occurring in an animal is not diagnosed in time, it may lead to the loss of the animal, or in the case of epidemics, it may spread between animals and cause mass animal losses. In health monitoring systems, the health status of animals is monitored with various sensors. Data such as heartbeat, body temperature, and blood pressure are collected through sensors and saved at regular intervals in a cloud-based storage unit via an internet protocol. Sensors are integrated into wearable devices such as collars and placed on the animal. The warning system ensures that information is conveyed to the farmer or the responsible veterinarian if any parameter in an animal deviates from normal. Additionally, the farmer can monitor at any time via a mobile or web interface. In this way, the problems of mass animal deaths and decrease in crop yield caused by diseases can be significantly reduced [34]. *Table 3* shows studies using IoT technologies in animal health monitoring.

Tracking Animal Location

To increase the productivity of animals, grazing animals must move around the livestock land. Especially in large feedlots, it is difficult for farmers to visually track the location of animals or requires intense physical strength. While grazing in open fields, an animal may not be able to return due to a snake bite or illness, and farmers may also encounter problems such as theft of animals. Technologies such as GPS used to track the location of animals allow determining where each animal is. Location tracking systems include methods that estimate the distance from the animal's current location to the barn's or farmer's location [57]. Thus, the farmer can see how far the animal is from the barn and set virtual boundaries for the animals. If the animal's distance to a key location, such as a barn, is over a certain unit, a notification can be sent to the farmer. This threshold unit can be defined relative to the boundaries of the farm, or a distance determined by the farmer. Geographically limiting animals will also prevent problems that may arise from animals entering another farm [19]. Studies on animal tracking systems are presented in *Table 4*.

Smart Feeding

Proper nutrition of animals is one of the factors that directly affect animal health and product productivity. Smart feeding systems include features such as monitoring the remaining food volumes in feedlots, monitoring feed

Table 3. *IoT applications in health monitoring*

Parameters	Sensors	MCU	Comm. Protocol	Critical Situation Decisive	Actuator	Animal	Ref.
Body temperature, heart rate, humidity	Accelerometer, body temperature (MLX90614) humidity (DHT11), heart pulse (KG011)	Arduino UNO	Bluetooth (HC05), Wi-Fi (HSP8266), IEEE 802.11 & 802.15	Threshold	N/A	Cow	[35]
Heart rate, rumination, body temperature, humidity	Temperature (DS18B20), Accelerometer for rumination (ADXL335)	Raspberry Pi3	IEEE 802.11 Wi-Fi standard	Threshold	Alert with GSM	Cow	[36]
Rumination, body temperature, heart rate, surrounding rate	Accelerometer for rumination (ADXL335), body temperature (TTC05102), heart rate (T56H), humidity (DHT11)	PIC18F455, XBee-PRO S2	Zigbee IEEE1451.1 standard	Threshold	Alert	Cow	[37]
Animal weight, number of approaches to the watering area, drinking time, and duration	There is a platform in the watering area to measure the weight	Arduino UNO	Wi-Fi	Threshold	N/A	Cattle	[38]
Temperature, acceleration, heartbeat, gas, sound, saliva, weight	Temperature sensor, accelerometer, heartbeat sensor, saliva sensor, gas sensor, microphone, load sensor	N/A	N/A	Fully connected neural network	Web-based monitoring	Cow	[39]
Temperature, humidity, heartbeat, rumination, respiration rate	Body temperature (LM 35), respiration, DHT 11 humidity sensor, heartbeat, and rumination	Arduino UNO	ESP8266 Wi-Fi	Threshold	N/A	N/A	[40]
Rumination sound, temperature, 3D movement	Rumination, temperature, motion sensors	Raspberry Pi 3	Wi-Fi	Artificial neural network	Alert to farmer	Cattle	[41]
Humidity, barometric levels, gyroscope, noise, GPS coordinates, heart rate, duration of activity, lying down	Video and thermal camera	N/A	LoRa and TCP, Wi-Fi	Boosted decision tree	Alert to farmer	N/A	[6]
Body temperature, humidity, heartbeat, position	Temperature and humidity (DHT22), heart rate (SON1205), gyro (GY-521 MPU-6050), Respiratory rate	N/A	HTTP	Light-gradient-boosting decision tree regression	Web-based monitoring	Cattle	[42]
Image, temperature, humidity, light	Temperature-humidity sensor: (SHT-71), luminance sensor (brightness sensor): GL5547	MCU (MSP430F1611)	ZigBee	Threshold	Water sprayer/fan, lighting	Cow	[43]
Animal movements, temperature, heartbeat	Temperature (LM 35), heart rates (infrared- IR sensor), postüre (ADXL 325)	MCU (16F877A)	Zigbee	Threshold	N/A	N/A	[44]
Temperature, heartbeat, location, pulse, respiration	Temperature (LM35), heart rate, pulse rate, respiratory, GPS	MCU	Zigbee. IEEE 802.15.4 standard	Threshold	Web-based monitoring	Pets	[45]
Environment temperature, humidity, light, CO_2 and NH_3 concentration Animal acceleration, rotation, body temperature, location	Temperature and humidity (AM2321), illuminance (GY-2561), CO_2 concentration (DFRobot SEN0219), NH_3 concentration (Winsen MQ137), acceleration (MPU-6050), GNSS, body temperature (DS18B20)	Raspberry Pi 3B, ATMega2560, M0 RFM95	LoRa low power wide area network (LPWAN)	Threshold	N/A	N/A	[46]

Table 3. *IoT applications in health monitoring (Continued)*

Parameters	Sensors	MCU	Comm. Protocol	Critical Situation Decisive	Actuator	Animal	Ref.
Environment temperature, humidity gas concentrations. animal temperature, heart rate, respiration rate, GPS, movement, activity	Air thermometer and humidity (SHT20), hygrometer, gas (CH4, H_2S, NH_3, CH20/H-CHO), body temperature, pulsometer, respiratory, accelerometer, gyroscope	Raspberry Pi, MCU (SoC)	Zigbee, Wi-Fi	Threshold	Web-based monitoring	N/A	[47]
Heart rate, body temperature, environment temperature, rumination, humidity	Infrared body temperature, 3-axis accelerometer, DHT 11 environmental temperature and humidity sensor	LPC 1313 MCU	LoRa	Threshold	Mobil based	Cow	[48]
Temperature, heart rate, weight, humidity	Temperature, load cell, humidity sensors	MCU	GPRS, Wi-Fi	Naive Bayes	Mobile-based alert	N/A	[49]
Rumen pH and temperature change	pH (TRY414.92) and temperature (DS18B20)	Arduino UNO R3	Serial Wi-Fi circuit	Fuzzy Logic	Web based monitoring	Cattle	[50]
Body temperature, heartbeat, animal falling	Temperature (LM35), heartbeat, piezoelectric sensors	AT89C51 MCU	ESP8266 Wi-Fi	Threshold	Mobile based alert	Cattle	[51]
Position, movement, heart rate	GPS (Pa1010D), heart rate (Maxin MAX30102), accelerometer and gyroscope (Adafruit LSM6DSOX)	M0 RFM95 MCU	LoRa, ESP8266, I2C	Threshold	E-mail alert	Horse	[52]
Heart rate, body temperature, acceleration	Heart rate, body temperature, inertial measurement unit	N/A	ESP 32 Wi-Fi, I2C	Threshold	N/A	Cow	[53]
Temperature, location, heart rate, water level, air quality, CO_2 concentration, environment humidity, temperature, light, virtual board	Temperature (DHT11), stethoscope (CR-747SS), ultrasonic (HC-SR4), air quality (MQ-135) sensors, GPS (NEO-6M)	NodeMCU	ESP8266 Wi-Fi	Threshold	Alert with GSM	Cow	[54]
Environmental temperature, humidity, CO_2, animal temperature, pulse	RFID, temperature and humidity (DHT11), CO_2 (MQ-135), body temperature and pulse sensors	Arduino	LoRa	Threshold	Turn on/off fans, open food, turn on/off massage, open/close Windows	Cow	[55]
Temperature, heart rate, oxygen separation level, respiratory range, position of the animal	SPO_2, temperature, humidity, respiratory, and heart rate sensors	Arduino UNO	Zigbee	Threshold	Alert with SMS, mobile app	Cow	[56]

Table 4. *Studies about animal location tracking*

GSM	GPS	GPRS	WSN	RFID	MCU	Comm. Protocol	Sensors	Actuator	Animal	Ref.
√	√		√		Arduino	XBee				[58]
	√	√					Ultrasonic sensor	Virtual board		[59]
			√	√	Raspberry Pi3	LoRa			Cattle	[60]
	√				MCU	LPWA network			Cow	[61]
	√				Arduino-compatible MCU	LoRa	Motion sensor (MPU-9250)		Grazing animals	[62]
	√				LPC 1313 MCU	LoRa		Virtual board	Cow	[48]
√		√		√					Cow	[63]

Table 5. *IoT applications in smart feeding*

Parameters	Actuators	Sensors/Hardware	Comm. Protocol	MCU	Animal	Ref.
Animal's feeding time, amount of feed in the silo, amount of feed in the warehouse, presence of cattle near the feed	N/A	Ultrasonic sensor, load cell, servo motor	Wi-Fi	Arduino WeMos D1R2	Cow	[66]
Activity, amount of feeding from the feeder and in the pasture, height of hay bales	N/A	Accelerometer (LIS3DH), GPS (Neo-6 m GPS sensor), ultrasonic sensors	RF, GPRS, Wi-Fi	Arduino	Beef cattle	[67]
Dog breed, size, and weight	Calculate the amount of feed the dog should take according to its breed, size, and weight.	DC motor	ESP8266 Wi-Fi	ATMEGA328 MCU	Dog	[68]
N/A	Automatic feeding in the aquarium	N/A	ESP8266 Wi-Fi	Arduino UNO, ESP 12F, ROHM IOT Kit control boards	Aquarium fish	[69]
Weight of bait bowl	N/A	Weight sensor	LoRa	Arduino UNO	Stray animals	[70]
N/A	Automatic feeding	N/A	Wi-Fi	N/A	Aquarium fish	[71]

stocks, monitoring temperature and humidity values in the feed that will reduce the quality of the feed, and protecting the feed in silos and warehouses from animals such as insects and rodents [64]. Animals are fed with ready-made feed in shelter areas and by grazing in open fields. Which feeding method and for how long the animals are fed is important in terms of monitoring the amount of food they receive. In this way, animal grazing areas can also be arranged [19]. Monitoring nutritional status with sensors helps maximize individual growth rates by ensuring that each animal receives the right quantity and quality of feed at the right time [65]. Studies on smart nutrition systems are summarized in *Table 5*.

Estrus/Calving Detection

In livestock farming, calving is important to increase or at least protect the number of animals on the farm. In dairy cows, the milk production efficiency of the animals depends on the calving intervals and calving between 12-14 months has a significant effect on lifelong milk production [72]. For this reason, the estrus periods of animals are monitored on farms. If the period cannot be detected, a 21-day non-pregnancy period occurs for the cows, and the fertilization period is disrupted. This situation causes a decrease in milk yield along with the cost of additional feed and artificial insemination for the cows [73].

Symptoms of animals' estrus periods include behavioral and hormonal changes. Behavioral symptoms usually manifest as irritability and restlessness. Animals may become anxious, avoid people, eat less, and make various sounds. Additionally, their number of steps may increase, and they may exhibit behaviors such as walking around other cows and sniffing. Hormonally, the animal's body temperature decreases before estrus and increases with the beginning of the period [74].

The estrus period for livestock lasts approximately 18 hours. Ovulation occurs within 8-11 h after the start of the period [75]. In traditional animal husbandry, this process is monitored by an observer. However, especially in large farms, situations such as the lack of a sufficient number of observers, the experience of the observer, the frequency of observation, falling at night or at a time when no one is on the farm may cause the estrus period not to be determined [76]. With the developments in smart animal husbandry, monitoring, and detection of estrus periods can be carried out automatically. While hormonal changes can be monitored through biosensors placed on the animals' bodies, changes in body temperature due to the estrus period and their movements can be monitored with accelerometers or cameras, thanks to wearable sensors. The data obtained is analyzed on IoT platforms and a notification is sent to the farmer or the responsible person via GSM or mobile application when the animal enters the estrus period. In this way, farmers are relieved of the obligation to make constant observations, and the risks associated with missing the period are also prevented.

Table 6. IoT applications in estrus/calving detection

Sensor/ Hardware	Parameters	MCU	Comm. Protocol	Animal	Actuator	Critical Situation Decisive	Ref.
Accelerometer	x, y, z coordination	Arduino UNO	Bluetooth (HC05), Wi-Fi (HSP8266), IEEE 802.11 & 802.15	Cow	Web-based monitoring	N/A	[35]
Infrared thermometer	Heat stress, body temperature	Arduino atmega 328p	Bluetooth, Wi-Fi (ESP8266)	Cow	N/A	N/A	[78]
Accelerometer (MMA7260)	Acceleration	MSP430 MCU	N/A	Cow	Web and smart-based monitoring	Expectation maximization, random forest, and CNN	[79]
IP camera (DH-SD22404T-GN)	Images and videos	Arduino UNO R3	Wi-Fi	Cow	Alert with GSM	Faster R-CNN	[80]
Digestible Biosensor (in rumen)	Internal body temperature	N/A	N/A	Cow	Alert with mobile app	N/A	[81]
Accelerometer (ADXL345)	Acceleration	ESP8266 Node MCU	N/A	Cow	N/A	N/A	[82]
Infrared thermometer (MLX90614ESF-BAA), accelerometer (MMA8451)	Step count, skin temperature	Wemos d1 mini (ESP8266EX)	Wi-Fi 802.11 b/g/n standards	Cow	N/A	N/A	[83]
Accelerometer (LIS3DH), GPS (Neo-6 m GPS sensor), vaginal thermometer	Location, movement, temperature	Arduino	RF, GPRS, Wi-Fi	Beef cattle	Mobile and web-based monitoring	N/A	[67]
Pedometer (afitag), activity meter (Heatime-RuminAct and HeatPhone)	Step count, calving date, lactation number, dairy production, progesterone in milk	N/A	Infrared connection, radio frequency	Cow	Alert	N/A	[84]
Pedometer	Step activity for estrus, lying time, and pregnancy stage for calving	N/A	MQTT	N/A	Alert with SMS	Random Forest, K-NN	[85]
Inertial measurement unit (IC-20948), GNSS, thermometer (DS18B20)	The number of transitions between lying and standing	nRF52840-dongle MCU	LoRa	Cow	N/A	N/A	[77]

Similarly, detecting the moment of birth of animals helps prevent the loss of dam and offspring by intervening early in various problems that may arise during birth. Therefore, in smart animal husbandry, the calving periods of animals are monitored by monitoring the animals' movements, behavioral changes, and body and hormone parameters. When the calving season comes, changes occur in the behavior of the animals. While there is a decrease in behaviors related to feeding and rumination, an increase can be observed in lying down. Behavior patterns and durations in animals can be monitored with sensors such as accelerometers and pedometers [77]. In *Table 6*, details of studies on IoT applications for estrus/calving detection are presented.

Lameness Detection

Lameness is one of the most common problems on livestock farms, after infertility and mastitis. Lameness, which reduces the fertility level and efficiency of milk production, especially for dairy cows, negatively affects the feeding and rumination processes of the animals [86]. Locomotor deficits and hoof and limb lesions are the main causes of lameness. In animals, behavioral changes such as slow movement, tendency to lie down, and lowering the head while walking determine this condition [3]. In traditional animal husbandry, these behavioural changes are detected observationally. However, this detection method can be subjective, time-consuming, and costly. Therefore, with the developments in smart animal husbandry, the

Table 7. *IoT applications in lameness detection*						
Parameters	**Sensors**	**Comm. Protocol**	**MCU**	**Animal**	**Critical Situation Decisive**	**Ref.**
Physical activity	Accelerometer	Bluetooth	ARM Cortex-M0 MCU	Cattle	One class SVM	[88]
Number of steps, lying time, and number of transitions between them (swap)	Pedometer	Message Queue Telemetry Transport (MQTT)	N/A	Cow	SVN, RF, K-NN, Decision tree	[14]

use of IoT systems for lameness detection is preferred. IoT systems both reduce observer costs and provide early diagnosis in detecting lameness. Early diagnosed lameness allows intervention to be made early, allowing savings in expenses such as antibiotics and veterinarians [14]. Generally, approaches to lameness detection are categorized into three groups according to the variables measured: kinetic, kinematic, and indirect measurement. In kinetic approaches, the forces involved in movement are evaluated; for example, the weight distribution and hoof strength of animals are examined during standing and walking. Kinematic approaches, on the other hand, focus on specific posture changes; They take into account variables such as the size, length, height, and back curves of the animals' steps. Indirect measurement examines behavioural changes; Values such as the animal's lying and standing time and milk yield are measured with the data obtained from the sensors integrated into the animals. Due to variations in measurement methods, even animals of the same species can exhibit different symptoms [87]. Studies about lameness are more limited than other fields, and the lameness detection studies discussed in this review are presented in *Table 7*.

Animal Activity Detection

Animals express their interactions with their environment through their behaviour. In situations such as illness, they go beyond their normal behavioural routines. So, behavioural changes can be a sign of the animal's health status. For example, if there is lameness, the animal's tendency to lie down increases, and during estrus periods, its steps increase. Feeding and rumination periods of animals are also among the values monitored in determining their health status. For a healthy animal, feeding and rumination periods are generally specific. Changes in these periods and durations may be a sign that there is something unusual in the animal. Therefore, monitoring the behaviour of animals is an important parameter in terms of their health status. Smart farming applications also include monitoring animal activities to monitor the

Table 8. *IoT applications in activity/behavior monitoring*							
Detected Activities	**Sensors**	**Parameters**	**MCU**	**Comm. Protocol**	**Animal**	**Critical Situation Decisive**	**Ref.**
Standing, lying, standing and ruminating, lying and ruminating, walking, walking and grazing	Temperature sensor, GPS (Neo-6M), 3-axis accelerometer (MPU 6050)	Temperature, acceleration on the x y, and z axes, latitude, longitude, and speed	ATMEL 328 MCU, Ardunio UNO	GSM (SIM 800)	Cattle	XGBoost, Random Forest	[90]
Standing, lying, walking	Pedometer	Step count, lying time, standing time, lying bouts, number of movements from lying down to standing up per hour	N/A	MQTT	N/A	Random Forest, K-NN	[85]
Grazing/eating, ruminating, neutral, walking, standing, lying	Inertial measurement unit (IC-20948)	N/A	nRF52840-dongle MCU	LoRa	Cow	Threshold	[77]
Walking, feeding, lying, and standing	3-axis accelerometer, GPS		Atmega328 MCU	LoRa	Cow	Random forest	[91]
Standing, lying, normal walking, active walking, standing up, and lying down	3-axis accelerometer (ADXL345)	Acceleration on the x y and z axes	MSP430 MCU	RF	Cow	Adaboost	[92]
Lying, standing	Ruuvitag sensor	Accelerometer, air temperature pressure, and humidity	Raspberry Pi	Bluetooth	N/A	N/A	[93]

animals' health status. The behaviour pattern monitored is determined depending on the application, but generally, the behaviours monitored are general behaviours such as walking, standing, lying down, and rumination. Sensors commonly used to detect behaviours exhibited by the animal include GPS, accelerometer, and step counter. In some studies, the activity of the animal is determined by classifying animal images collected with cameras. Additionally, by taking sound data from animals through microphones, behaviours such as grazing and rumination can be detected. It is also used for situations such as determined activities of animals, estrus, birth, and lameness detection [3,23,89]. Studies conducted in this field are given in *Table 8*.

Fish Farming

Depending on changing climatic conditions, ecological balances in nature also change. These changes bring about a decrease in water levels and an increase in water pollution. The demand for fish in the world's increasing population cannot be met by natural breeding methods [94]. For this reason, fish farming in farms is becoming increasingly common.

Water quality is a factor that directly affects the health and survival of fish. Dissolved gases in water, such as carbon dioxide, ammonia, and oxygen, affect water quality. Increasing the concentration of dissolved carbon dioxide can cause oxygen levels to drop, which can lead to fish suffocation. The increase in ammonia concentration causes fish poisoning. The higher the dissolved oxygen concentration in the water, the better the water quality. The amount of oxygen dissolved in water also depends on the salt level and temperature of the water. The recommended optimal water temperature for fish is 25-27 degrees, which may vary depending on the type of fish grown [69,95]. High or low pH is dangerous as it will cause fish to become sick, poisoned, or unable to grow. The recommended appropriate pH value is between 6.5-8.5. Certain water levels must be maintained for fish to survive. High volumes of precipitation and flooding cause ponds to overflow, which can cause fish to flush out. The increase in air temperatures will reduce the water level due to excessive evaporation [96].

Nowadays, fish farms can be monitored remotely thanks

Table 9. *IoT applications in fish farming*

Parameters	Sensors/Hardware	Actuators	Comm. Protocol	MCU	Ref.
Water temperature, depth, dissolved oxygen value, air pressure	Infrared distance (GP2Y0A02YK), pH (SEN0169), dissolved oxygen (SEN0237-A), pressure (BME280) sensors	N/A	Wi-Fi ESP8266	MSP430G2553 MCU	[95]
Temperature, pH, electrical conductivity, dissolved oxygen, total dissolved solids, water level	Temperature, pH, electrical conductivity, oxygen, dissolved solids water level sensors	Food feeder, pump, heater, fan, light, UV, RO (reverse osmosis)	Wi-Fi ESP8266	Arduino UNO, ESP8266 ESP 12F, ROHM IoT Kit control boards	[69]
pH value, salinity, and temperature of water	pH, salinity and temperature sensors, servo motor	Lowering or raising the pH	N/A	Arduino, Raspberry Pi	[97]
Temperature, water level, oxygen, pH	Temperature, oxygen, pH sensors	Water pump, fish feeder, pond heater lighting led	Wi-Fi, Zigbee	N/A	[94]
Temperature, pH value, dissolved oxygen, water level, and life expectancy	Temperature, pH, dissolved oxygen sensors	Alert to farmer	LoRa	Arduino Mega 2560 MCU	[96]
Water temperature, water level, light	Temperature (DS12B20), light intensity (RTC-DST1302), water level sensors	Heater, Buzzer, RGB Led, LCD Display, Led light	Http, wivity module	Arduino Mega 2560	[98]
pH, dissolved oxygen and ammonia levels, water temperature	pH, CO_2, NH_3 and temperature (DS18B20) sensors	Alert via mobile phone	ESP-12E Wi-Fi	Node MCU	[99]
pH, water temperature, water level	pH, temperature (LM35), water level sensors, DC motor	PC based monitoring	Wi-Fi	ARM LPC 2148 MCU	[100]
Water temperature, electrical conductivity, level, pH value, body turbidity and dissolved oxygen	Temperature (DS18B20), electrical conductivity (TDS), water level, pH (E-201-C), water body turbidity (Eater WT-RCOT), and oxygen (YHT-8402) sensors	Water supply and drainage pumps, aerators, feeders, water filtration purifiers	LoRa, GPRS	N/A	[101]
Water temperature, turbidity, pH, water level, CO_2	Water temperature, turbidity, pH, water level, CO_2 sensors	N/A	ESP 8266 Wi-Fi	Ardunio	[102]

to IoT systems. In this way, environmental factors are monitored in closed fish farming areas and productivity is increased by keeping the parameters of these factors at optimal levels. With this method, profits are maximized by reducing costs and increasing product quantity. Thanks to sensors that measure values such as temperature, pH, and dissolved oxygen integrated into water tanks, growers can instantly monitor the water values via mobile or web-based applications. In addition, various actuators are activated according to the data coming from the sensors, and automatic intervention can be made when water parameters such as temperature and gas go beyond safe limits. *Table 9* presents studies on IoT technologies developed/used for fish farming.

Smart Beekeeping

Bees are vital to environmental well-being and provide pollination, which is essential in agriculture. Bee pollination provides far-reaching benefits to food processing, raw materials, pharmaceuticals, plants, social and cultural values, and the maintenance of biodiversity and environmental protection [103]. Although all bee species contribute to pollination, honeybees are the primary pollinators of almonds, citrus fruits, blueberries, and cucurbits. Therefore, any changes in bee colony behavior and health, as well as declines in population sizes, can have dramatic impacts on the food industry. For this purpose, constant monitoring of bee health and colony strength is carried out by beekeepers. Limitations of manual observation include the difficulty for beekeepers to regularly monitor bee colonies and assess their health status. For this reason, there is an increasing trend towards new technologies that automate and extend hive monitoring technologies.

Humidity and temperature levels in the hive are important for the queen's egg production, larvae rearing, and food preservation. In smart beekeeping systems, hive values can be monitored and controlled remotely with temperature and humidity sensors [104,105]. Colony weight is one of the oldest monitored hive indicators of hive health, colony workforce, and hive food stores. Weight scales are more expensive than sensors and require more complex setups [106]. For a professional beekeeper managing hundreds or thousands of hives, adding a weight scale to each hive would be a huge expense. Therefore, recently beehive weight scales, which can automatically send periodic data to data storage using IoT and SMS technology, have gained popularity for continuous monitoring [107-109]. Pests and diseases always pose a threat to beehives. This can cause the rapid collapse of entire hives, a phenomenon called Colony Collapse Disorder (CCD) [110]. To mitigate the potential effects of these devastating pest and disease outbreaks, disease and pest control can be carried out in beehives with IoT systems. Image sensor-based approaches [111] and gas sensor-based approaches [112] involving thermal imaging are used in honey bee hives. Audio and imaging techniques called hive health monitors are also used to monitor beehives. Studies discussing hive health in terms

Table 10. *IoT applications in beekeeping*

Parameters	Sensors/Hardware	Duty	Comm. Protocol	MCU	Critical Situation Decisive	Actuator	Ref.
Bee sound	Audio receiver	Swarm activity classification	N/A	N/A	Deep neural network	N/A	[113]
Temperature, humidity, pressure, beehive weight	Temperature (DHT 11), LDR light, weight sensors, servo motor	Remotely monitoring and controlling the status of hives	LoRaWAN	ATmega32u4 MCU	N/A	Web-based monitoring, LED diode for status signaling, SG90 Micro Servo actuator	[108]
Temperature, humidity, light intensity, and rain level	N/A	A prediction and early warning system for the population daily loss rate of bee colonies	UDP socket	Raspberry Pi 3	Temporal convolutional network	Mobile based monitoring	[114]
Temperature and humidity	Temperature and humidity (DHT22), sound (MAX4466 amplified) sensor	Colony health monitoring	TCP/IP, ESP8266, local Wi-Fi network	NODE MCU, Arduino ATmega2560	N/A	API, Web-based, mobile based	[115]
Video, temperature, humidity, weight, and audio	Video, audio, temperature and humidity AM2302, weight sensors	Warning and monitoring system to prevent significant losses in the population of hives.	FTP, MQTT SSH	Raspberry Pi 4	N/A	Web-based monitoring and alert	[116]

of hive characteristics generally include brood size and/or dynamics, forager workforce size and/or dynamics, hive internal environment, hive audio, hive resources, and pathogen infestation. IoT studies conducted in the field of beekeeping are summarized in *Table 10*.

Smart Poultry

People consume poultry extensively. Chicken meat is the most preferred among poultry. As the demand for chicken meat increases globally, so do poultry quality concerns. Modern technological advances are helping the poultry industry in monitoring chicken health [117]. These advances include detecting the disease and health of chickens using video surveillance, audio observations, and IoT-based wearable sensor devices. These devices are placed on chickens and/or in coops and used for further analysis.

The use of modern advances provides the opportunity to monitor and early detect chicken diseases in poultry farms. These chicken monitoring techniques may include vocal analysis [118], which can automatically monitor the chicken's behaviour without direct interaction with the chicken's body. Wearable sensing devices [119] help determine the location of chickens and automatically identify and track their real-time movement with radio frequency identification devices. Surveillance of chicken farms through image processing [120] is another technological advance to identify activity behaviours and detect disease early. Diseases such as the H5N1 bird flu virus in poultry cause the death of animals but also affect human health. However, since chickens are housed in very close proximity to each other in coops, a viral disease can spread very quickly. Monitoring the body temperatures of animals is effective in taking early precautions in case of an epidemic [121]. The main goal of chicken breeders is to ensure maximum growth by maintaining the optimal weight of the animal. Chickens should gain weight quickly, but since this also restricts egg laying, it must be kept in balance. Therefore, the amount of food consumed by chickens can be determined according to their weight.

The environmental conditions of the poultry environment have a direct impact on productivity. These environmental inputs can be temperature, humidity, and concentration of various gases such as carbon dioxide and ammonia. Environmental parameters such as temperature (house temperature should be 20-26°C), humidity (should be approximately 50-70%), ammonia gas (should be below 10 ppm), and light are environmental conditions that directly affect chicken welfare. When concentrations of ammonia and carbon dioxide gas in the air increase, it can cause

Table 11. *IoT applications in poultry*

Parameters	Sensors/Hardware	Duty	MCU	Comm. Protocol	Actuator	Critical Situation Decisive	Ref.
Temperature, humidity, ammonia concentration, light, luminosity	Temperature and humidity (DHT22) Electrochemical (MQ-137) LDR sensors	Monitoring environmental conditions for poultry houses	*Wemos* Mini D1 MCU	ESP826, Wi-Fi	Mobile based monitoring	Least Squares Method (MMQ)	[126]
Temperature, humidity, gas	Temperature, humidity, gas (CO_2, O_2, NH_3) sensors	Monitoring environmental conditions for poultry houses	Raspberry Pi	Sim900 GSM, WSN	Mobile alerts, dashboard	N/A	[127]
Pecking, Preening, Dustbathing	RFID microchips and accelerometers	Disease monitoring for chickens	RFID microchips	Radio Frequenc, CSMA	Web-based monitoring	Decision Tree, Logistic Regression, KNN, Gaussian Naive Bayes, RF, SVM, TabNet	[128]
Temperature, humidity, gas	Temperature and humidity (DHT11), gas (MQ135) sensors	Monitoring environmental conditions for poultry houses	Raspberry Pi	Inter-integrated Circuit (I_2C), Wi-Fi	Web-based monitoring	N/A	[129]
Temperature, humidity, light, CO_2, ammonia and hydrogen concentrations	Temperature, humidity, light, CO_2, NH_3, and hydrogen sulfide concentration sensors	Monitoring environmental conditions for poultry houses	N/A	Zigbee, GPRS	Alert, ventilate, light, temperature, humidity,	N/A	[130]
CO_2, NH_3 concentration, poultry temperature	CO_2 and NH_3 concentration, temperature, and humidity (DHT22) sensors	Monitoring environmental conditions for poultry houses	N/A	UTC-4432B1 wireless module	N/A	N/A	[131]

vision difficulties in animals, affect the respiratory system, and eventually cause death [122]. Especially during breeding periods, the temperature in the chicken coop is desired to remain within the recommended range [123]. Because hot or cold can cause stress in animals. Low relative humidity increases the rate of heat dissipation through evaporation and negatively affects the performance of animals [124]. Animals are sensitive to light. Exposure to more or less daylight than recommended affects nutritional intake and sexual maturity in chickens [125]. To monitor and improve environmental conditions, developed IoT-based systems are given in *Table 11*.

Discussion

In general, livestock management systems consist of three distinct processes: sensing and monitoring, analysis and decision-making, and intervention. PLF systems facilitate the management of these processes by reducing the need for manual observation and human-based decision-making. In traditional animal husbandry, all of these processes rely on human labour. However, in IoT-based systems, sensors, cameras, microphones, and other hardware are utilised for sensing and monitoring, machine learning algorithms are employed for analysis and decision-making, and automatic actuators are activated for intervention. This significantly reduces the time and labour costs required to manage a large number of livestock. Furthermore, these systems, when established with correct parameters, hardware, and algorithms, can prevent losses resulting from human negligence or error. Consequently, producers are enabled to manage more animals with fewer maintenance costs.

IoT applications in animal husbandry are becoming increasingly popular. This technology offers farmers a range of advantages. Firstly, IoT provides real-time data by monitoring the behaviour, health status, and environmental conditions of animals, thereby offering valuable insights for farmers to make more efficient and effective decisions. For instance, receiving early warnings about the health status of animals enables early diagnosis and treatment of diseases, thus reducing animal losses and veterinary expenses. Secondly, IoT enables more efficient use of resources. Resources such as feed and water consumption become traceable and manageable, leading to savings for farmers and enhancing environmental sustainability. Additionally, IoT automates and facilitates business processes. Providing remote access and automatic control increases labour productivity and enables farmers to manage their time more effectively. Moreover, IoT aids in predicting future trends through the analysis of collected data, providing farmers with a valuable tool for making strategic decisions. Therefore, IoT applications play a significant role in the livestock sector and are expected to become even more widespread in the future.

Due to the numerous benefits of IoT technology in animal husbandry, applications are being developed in various areas, and there has been an increase in academic research in this field in recent years. Although there are some studies on IoT in PLF systems in the literature, their scope is more limited. They focus on examining equipment that may be needed within an IoT system, such as a single animal species like chickens or fish [5,23,133,135-139], a few application areas like animal health monitoring [132-139], or technologies used in the scope of IoT [4,137]. However, in this compilation, a much more comprehensive review of IoT application areas in animal husbandry has been conducted without being limited to specific animal species. Additionally, information regarding the technologies needed in the development phase of an IoT-based smart livestock system has been provided.

Studies generally focus on monitoring the health of animals based on values such as body temperature and heart rate. However, it is observed that sensors like GPS, pedometers, and accelerometers are widely used in various application areas. In most studies, suitable threshold values for parameters have been defined, and warning systems have been developed based on data from sensors. Deviation from the specified threshold values in sensor data can pose a problem. It is expected that the use of machine learning algorithms that can predict based on past data and provide warnings before critical situations occur will become more widespread in the near future. Additionally, as observed from the reviewed studies, the scope of actuators, an important component of an IoT system, is generally limited to providing notifications. With the increasing use of IoT systems, the proliferation of automatic systems capable of problem-solving or providing pre-solutions without human intervention is also expected.

When examining IoT applications in animal husbandry and the compilation of articles in this field, it is noted that most studies will be conducted in the near future. This indicates that IoT technology is rapidly advancing in the livestock sector and that research and applications in this area are increasing. These recently conducted studies highlight the potential and importance of IoT technology in animal husbandry. However, the limitations of research in this field and future studies that need to be conducted should also be considered. Specifically, further research and assessment are required regarding the cost-effectiveness, reliability, and efficacy of IoT applications in animal husbandry. Furthermore, the broad-ranging effects of IoT technology on the livestock sector and its potential contributions to sustainability are worth further investigation. In this context, it is expected that future studies will focus on further enhancing the applications of IoT technology in animal husbandry and increasing efficiency in the sector.

With the concepts of the IoT, traditional animal husbandry has begun to be replaced by smart farming. This change helps solve problems such as labour costs commonly encountered in traditional agriculture. For smart farming systems to operate efficiently, there are trends such as data collection and analysis, health monitoring and management, and the control of environmental units. Data are collected through wearable devices and sensors in the environment. This data is stored in cloud environments or local storage areas and processed using extensive data analysis methods or artificial intelligence. In this way, the health of the animals, their location and nutrition, and the welfare of their living environment can be monitored. Additionally, IoT sensors can optimise waste management.

While smart livestock systems offer many benefits, they also bring various challenges. The most important of these are data privacy and security. Since sensor data is transmitted over wireless systems like Bluetooth and radio frequency, it is more vulnerable to cyber-attacks. Additionally, various measures and monitoring must be implemented to ensure the privacy of the stored data. IoT systems particularly require financial investment during the initial setup phase due to the need for sensors, wearable devices, software, and data communication. After the setup, the maintenance and updates of these systems necessitate continuous investment. The typical establishment of livestock farms in rural areas, away from city centres, can lead to connectivity issues and technological infrastructure problems. To maximise the benefits of smart farming systems, farmers must adapt to the relevant technologies. Overcoming these challenges and ensuring continuous development is crucial for deriving maximum benefit from these technologies.

Conclusion

PLF systems, powered by IoT technology, offer significant benefits in optimising livestock management. By automating sensing, monitoring, analysis, and decision-making processes, PLF systems reduce reliance on manual labour, thus enhancing operational efficiency and reducing costs. IoT applications enable real-time monitoring of animal health, behaviour, and environmental conditions, empowering farmers with valuable insights for informed decision-making. Furthermore, IoT facilitates resource optimisation, business process automation, and future trend prediction, leading to improved productivity and sustainability in the livestock sector.

Although IoT technology in animals is rapidly advancing, further research and development are necessary to explore broader IoT integration and enhance cost-effectiveness and sustainability in managing livestock. Future studies should focus on addressing the reliability and efficacy of IoT applications and their potential contributions to sustainability. Additionally, advancements in machine learning algorithms and proactive problem-solving automatic actuators hold promise for further enhancing the efficiency of IoT-based livestock management systems.

Overall, the comprehensive review of IoT applications highlights its transformative potential in livestock farming. With ongoing advancements and interdisciplinary collaboration, IoT-enabled PLF systems are poised to drive sustainable improvements in productivity, animal welfare, and resource utilisation within the animal industry.

Declarations

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Research Article

Efficacy of Filgrastim in Canine Parvoviral Enteritis Accompanied by Severe Leukopenia

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Abstract

Canine parvoviral enteritis (CPE) is a common, highly contagious viral disease characterized by severe hemorrhagic gastroenteritis in dogs. Leukopenia and neutropenia in dogs with parvoviral enteritis are considered negative prognostic indicators. This study aimed to investigate the effect of filgrastim [recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF)] on leukocyte counts in dogs with parvoviral enteritis. The animal material consisted of thirty-seven owned dogs with parvoviral enteritis from various breeds, which were brought to the Small Animal Clinic of the Department of Internal Medicine, Faculty of Veterinary Medicine, Erciyes University. The dogs included in the study were divided into two groups. The first group received standard treatment (ST=18) and the second group received Filgrastim (ST+Filg=19) in addition to the standard treatment. For the assessment of leukocyte counts, blood samples were taken on day 0 (before starting treatment), day 3, and day 5 after treatment. It was observed that 16 out of the nineteen dogs treated with Filgrastim in addition to standard treatment (84.21%) showed improvement. In the ST+Filg group, the median WBC and neutrophil values on the 5th day after treatment were significantly higher than on the 0th day (P=0.001, P=0.006, respectively). In addition, the median WBC, lymphocyte and neutrophil values of the dogs in the ST+Filg group on the 3rd and 5th days after treatment were found to be significantly higher than the same day measurements of the ST group (P<0.001). As a result, this study determined that filgrastim (r-metHuG-CSF) contributed positively to the improvement of leukopenia in dogs with CPE, in conjunction with clinical recovery. It was concluded that filgrastim (r-metHuG-CSF) may be included in treatment protocols as one of the immunostimulant drugs to increase leukocyte counts in the treatment of dogs with parvoviral enteritis.

Keywords: Canine parvoviral enteritis, Dog, Filgrastim, Leukocyte, Treatment

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Introduction

Canine parvoviral enteritis (CPE) is a viral disease in dogs that is common, highly contagious, and characterized by severe hemorrhagic gastroenteritis [1]. *Canine parvovirus* (CPV) was first identified in the late 1970s and has since become a panzootic condition affecting dogs of all ages, primarily affecting puppies [2]. In 2022, the International Committee on Taxonomy of Viruses (ICTV) classified CPV in the *Parvoviridae* family, *Parvovirinae* subfamily, and *Protoparvovirus* genus [3]. CPV is an enveloped virus with a single-stranded DNA. *Canine parvovirus* type 2 (CPV, *Proparvovirus carnivoran* 1) and its variants, CPV-2a, 2b, and 2c, are responsible for the disease [4]. Although dogs of all ages can be affected, the disease is more common in puppies with underdeveloped immune systems and defense mechanisms (6 weeks to 6 months) [5]. The rapid replication of the virus targets cells with the ability to divide since viral replication occurs during the S phase of cell division. Therefore, puppies are more severely affected than older dogs [6]. If the animals are left untreated, CPE has an approximate mortality rate of about 91% [7]. In CPE cases, death is often associated with severe sepsis and endotoxemia and electrolyte abnormalities [5,8,9].

Typically, the prognosis of an illness is determined by the severity of clinical and laboratory data at the onset of treatment. With an appropriate treatment regimen, high mortality rates can significantly be reduced [5,10]. Clinical symptoms of CPE include lethargy, vomiting, fever, diarrhea, and anorexia. As the disease progresses, diarrhea becomes foul-smelling and bloody. High fever, severe vomiting, and bloody diarrhea result in a significant loss of fluids and blood from the body. Especially in puppies, this fluid loss can lead to death in a short time [1].

Abnormal laboratory findings in CPE include leukopenia, lymphopenia, neutropenia, and anemia [1,4,11,12]. This hematological outcome contributes significantly to the impairment of immune function. As a result of viral infection, the thymic cortex collapses and disappears. This causes substantial leukopenia in infected animals, along with the death of leukocyte precursors in the bone marrow [1,8]. Dogs that die from CPE generally have a total leukocyte count equal to or less than 1030 cells/μL, and it has been reported that persistent lymphocytopenia, monocytopoenia, and eosinopenia are observed within the first 3 days after hospitalization [4]. According to Goddard et al.[13] after admission and the first 48 h of hospitalization, total leukocyte counts above 4500/μl and lymphocyte counts exceeding 1000/μl have a substantial impact on survival.

In addition to acid-base and electrolyte imbalances, hydration and balancing oncotic pressure are important aspects in the treatment of CPE. Antiemetics, broad-spectrum antibiotics, probiotics, vitamins, minerals, amino acids, antiviral drugs, and immunostimulants are among the other drugs used in treatment of CPE [14,15]. Symptomatic treatment, supported by immunomodulators, cytokines, interferons, and antioxidant substances, significantly reduces mortality rate resulting from this disease [1,16,17]. The hematopoietic regulatory glycoproteins known as granulocyte colony-stimulating factors (G-CSF) facilitate the growth, maturation, and stimulation of neutrophils within the bone marrow [18]. The use of human granulocyte colony-stimulating factor (hG-CSF) in dogs has been reported to stimulate bone marrow and neutrophil release [16-18]. One form of colony stimulating factor is filgrastim, which belongs to a class of drugs that promote blood cell formation and function [19]. White blood cell production can be enhanced by the naturally occurring protein called G-CSF. Filgrastim is a man-made version of G-CSF that stimulates white blood cell production, particularly neutrophil production. Filgrastim prevents neutropenia associated with cancer treatment in human medicine [20]. Moreover, it can be utilized to increase white blood cell counts prior to stem cell extraction for transplantation [20,21]. Filgrastim is also used in dogs and cats to treat severe neutropenia [22].

In recent years, Punia et al.[23] in dogs with hemorrhagic gastro-enteritis, Areshkumar et al.[24] in 1 CPE positive dog, and Gülersoy et al.[17] in CPE-positive dogs without taking leukopenia levels into account, used human-specific granulocyte-colony stimulating factor (rcG-CSF) and provided useful information about its effectiveness. Additionally, Duffy et al.[16] in parvovirus induced neutropenia and Armenise et al.[18] in CPE-positive animals with leukocyte counts below 3000 (cell/μL) investigated the effectiveness of dog-specific granulocyte-colony stimulating factor (rcG-CSF). In the current study, unlike similar studies conducted to date, only dogs with leukopenia were used and it was aimed to investigate the effect of human-specific granulocyte-colony stimulating factor (rcG-CSF, Filgrastim) on total leukocyte, lymphocyte and granulocyte levels in a total of 19 CPE-positive dogs.

Materials and Methods

Ethics Statement

The Erciyes University Animal Experiments Local Ethics Committee accepted this work, and a certificate of approval (Decision date and no: 07.09.2023/169) was acquired.

Animals

Canine parvoviral enteritis (CPE) was diagnosed in sixty dogs brought to the Erciyes University Faculty of Veterinary Medicine, Department of Internal Medicine, Small Animal Clinic, Infectious Diseases Unit during the study period. Thirty-seven of these 60 dogs with CPE had significant leukopenia (including lymphopenia and neutropenia) on complete blood count analysis. Eighteen out of the thirty-seven dogs were included in the Standard Treatment (ST) group. Nineteen out of thirty-seven dogs were included in the Standard Treatment + Filgrastim (ST+Filg) group. Filgrastim (r-metHuG-CSF) was administered to the dogs in the ST+Filg group as an immune stimulant drug in addition to the standard parvoviral enteritis treatment in order to increase their leukocyte counts. Dogs with CPE included in the study were pursued for 5 days. After the 5th day, these dogs were continued with an additional treatment protocol if deemed necessary. This additional protocol included supportive treatment practices such as dietary supplements, probiotics, anti-anemic medications and vitamin supplements. Blood samples were taken from these dogs on day 0 (before treatment) and on days 3 and 5 after treatment *(Fig. 1)*. During the treatment, these dogs were brought to the treatment daily by the pet owners

Owned dogs between the ages of six weeks and six months, with or without a history of vaccinations, exhibiting the typical clinical symptoms of canine parvoviral enteritis (vomiting and either hemorrhagic or non-haemorrhagic

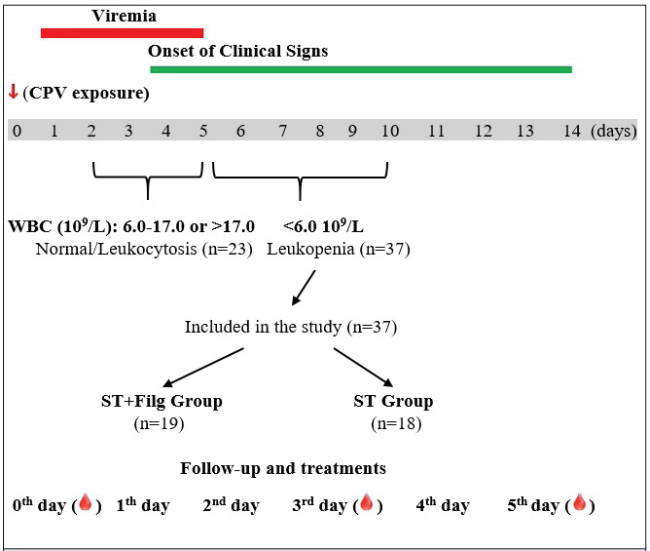

Fig 1. Study design and number of dogs included in the study. ST: Standard treatment, ST+Filg: Standard Treatment + Filgrastim

diarrhea), testing positive for CPV antigen in feces using a test kit and positive PCR test results, and having marked leukopenia (<6.000x10⁹/L) in a complete blood count analysis were the criteria for inclusion in the study.

Physical Examination

Every dog involved in the research was given a thorough physical examination. These examinations were performed by a single individual. Parameters such as body temperature, heart rate, respiratory rate, and blood pressure were measured. The degree of dehydration (<5% [subclinical], 5% [mild], 6%-8% [moderate], 8%-10% [severe], ~12% [hypovolemia]) was estimated using parameters such as skin elasticity, capillary refill time, dryness of mucous membranes [25].

Using the Rapid Diagnostic Kit for Antigen Detection

In suspected cases of canine parvoviral enteritis based on the clinical examination, lateral flow immuno-chromatographic (LFI) rapid diagnostic kits for CPV antigen (Anigen Rapid CPV antigen test kit Bionote®, BIONOTE Inc., South Korea) were employed. Before analysis, all kit materials were brought to room temperature, and the test platform was placed on a flat surface. Rectal fecal samples were obtained from the dogs using sterile swabs. These swab samples were then immersed in a tube containing sample buffer and mixed for 10 sec to ensure homogenization. Subsequently, the tube was placed on a flat surface and left for 3 min. After the particles settled at the bottom of the tube, the content was aspirated from the upper part of the tube. Then, three drops of the sample were dispensed into the sample well. The results were observed within 5 min. If a single line was visible in the observation window, the result was considered negative. If two lines were visible, the result was considered positive.

Polymerase Chain Reaction (PCR) Analyses

After collecting fecal samples from dogs showing clinical symptoms and testing positive with the LFI, the samples were processed at the Erciyes University, Faculty of Veterinary Medicine Virology Laboratory. To prepare the samples for DNA extraction, a portion of the feces was mixed with sterile 1x phosphate-buffered saline (PBS) solution containing 1% penicillin-streptomycin inside a biosafety level 2 (BSL-2) cabinet. The mixture was then passed through 0.22 µm syringe filters.

For DNA extraction, an optimized phenol-chloroform-based method was employed in the laboratory. In brief, 500 µL of the sample was mixed with 250 µL of phenol and 250 µL of chloroform/isoamyl alcohol (24/1) and vortexed. After centrifugation at 10.000 rpm for 10 min, the upper phase (400 µL) was transferred to a new microcentrifuge tube. Then, 3M sodium acetate (pH 4.2) at 1/10 volume and absolute ethanol at 2/10 volume were added, followed by vortexing.

The sample was incubated at -80ºC for 1 h and then centrifuged at 10.000 rpm for 10 min. The supernatant was removed, and 500 µL of 70% ethanol was added, followed by vortexing. The supernatant was disposed of following a second centrifugation cycle at 10.000 rpm for 10 min. The remaining DNA pellet was air-dried on filter paper. Finally, 20 µL of nuclease-free water was added, and the DNA was stored at +4ºC for use in the PCR process.

For PCR, a DNA extraction mix was prepared with 5 µL of DNA template, 1 µL each of forward and reverse primers (10 pmol), 1 µL of 10 mM dNTPs, 5 µL of 10x PCR Taq buffer (Transgen Biotech, AP111-01), 0.5 µL of Taq DNA polymerase enzyme (2.5 U), and 36.5 µL of nuclease-free water as the final volume. The primers used were specific to the VP2 gene of the virus: F: 5'-GCTGAGGTTGGTTATAGTGCR-3' and R: 5'-TGGATTCCAAGTATGAGAKGCT-3'. After preparing the samples for PCR, they were placed in a thermal cycler. The PCR program consisted of an initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 52ºC for 1 min, and extension at 72ºC for 1 min. A final extension step was carried out at 72ºC for 10 min. The PCR amplification products were run on a 1.7% agarose gel at 120V for 30 min in an electrophoresis system. The results were visualized under UV light.

Blood Pressure Measuring

Non-invasive blood pressure measurements were obtained using the PetTrust oscillometer (PetTrust, BioCARE, Taiwan). To minimize stress during blood pressure measurements in dogs and help them acclimate to unfamiliar personnel, a 5-10 min waiting period was

observed. During the blood pressure measurements, the animal owner, assisting staff, and the veterinarian conducting the examination were present. With the use of the measuring tape that came with the PetTrust, the cuff size was established. To select the appropriate cuff size, the circumference of the dog's right forelimb (proximal) was measured with the assistance of a flexible measuring tape. Approximately 30% of the circumference of the right forelimb was used as the standard for selecting the appropriate cuff size. The available cuff sizes were 2.05, 2.55, 3.05, 3.55, 4.05, 4.55, and 5.55 cm, respectively.

To measure blood pressure in the proximal part of the right forelimb, the cuff's bladder was placed over the arteria radialis in the middle of the antebrachium, ensuring that it was neither too tight nor too loose. When the device was activated, the cuff automatically inflated. The oscillometric measurement method was used to measure the mean arterial pressure (MAP), diastolic blood pressure (DBP), and systolic blood pressure (SBP). The results were then recorded. All measurements were conducted by an experienced veterinarian. The initial blood pressure measurements were disregarded. Subsequently, a total of three measurements were taken with a 15 sec interval between consecutive readings. The arithmetic average of these three readings was used for data analysis.

Blood Sampling and Complete Blood Count Analysis

After the clinical examination of the dogs (before starting the treatment), blood samples were collected from the cephalic veins of the dogs for hematological examinations to determine the infection status and leukocyte counts within the standard practices of the clinic. Subsequently, blood samples were collected again on the 3rd and 5th days after the initial treatment. Blood samples were duly taken into BD Vacutainer® K_2 EDTA tubes (Becton Dickinson, USA) for hematology analyses. Blood samples were delivered to the lab (Erciyes University, Faculty of Veterinary Medicine, Animal Hospital, clinical hematology, and biochemistry laboratory). Hematological analyses of the dogs with parvoviral enteritis were performed using a complete blood count device (Exigo EosVet, Boule Medical AB, Stockholm, Sweden) in the laboratory. Blood samples taken into BD Vacutainer® K_2 EDTA (Becton Dickinson, USA) tubes were homogenized for 3 min at 40 rpm/min using a roller blood mixer before analysis.

Treatment

In order to administer fluid-electrolyte therapy, the ill dogs' level of dehydration was assessed. For the purpose of fluid-electrolyte therapy, the following were administered: lactated ringer (Polifleks, Polifarma), 5% Dextrose (Polifleks, Polifarma), isotonic 0.9% NaCl (Polifleks®, Polifarma), Dextran 40 (Poliflex 100 mg/mL Dextran 40 + 9 mg/mL Isotonic Sodium Chloride solution for IV infusion, Polifarma) alone or in combination. Maintenance fluids were supplemented with potassium chloride (0.05-0.4 mEq/kg/h) for sustaining normokalemia or restoring hypokalemia. The total amount of fluid to be given was calculated according to the formulas [25]: Fluid deficit (L) (body weight [kg] X % dehydration/100) + maintenance needs (40-60 mL/kg per day) + ongoing losses (1 mL/kg per h).

Ampicillin-sulbactam (SULCID® 0.5 g IM/IV, İbrahim Etem Ulagay İlaç Sanayi Türk A.Ş.) was administered at a dose of 10-30 mg/kg, IV, q 6-8 h. An intravenous injection of vitamin C (Vitce®, Sanovel) at a dose of 200 mg/kg was given for five days. Amino acids, vitamins and electrolytes for extra energy (Duphalayte®, Zoetis) was administered in a practical dose of 10 mL/kg for 5-7 days. Metoclopramide (Metpamid®, Sifar İlaçları Tic. ve San. A.Ş.) was used at a dose of 0.5 mg/kg. In persistent vomiting cases, maropitant citrate (Cerenia®, Zoetis) was also injected subcutaneously once a day at a dose of 1 mg/kg. *Bacillus clausii* (Enterogermina, Sanofi-Aventis SpA) was administered orally at a dose of 2 mL for 3-5 days as a probiotic. Hyoscine butylbromide (Buscopan® 20 mg/mL Injectable Solution, Sanofi İlaç San. ve Tic. A.Ş.) was administered by intramuscular injection daily twice at a dose of 0.5 mg/kg as an antispasmodic drug. In addition to standard treatment in the ST+Filg group, Filgrastim (recombinant methionyl human granulocyte colony-stimulating factor [r-metHuG-CSF] [Fraven 30 MIU/0.5 mL, IV infusion/SC Injection, Arven İlaç San.]) was administered subcutaneously at a dosage of 10 µg/kg once a day for 5 days. This dose of filgrastim (r-metHuG-CSF) was chosen in accordance to the study performed by Punia et al.[23] and Areshkumar et al.[24].

Statistical Analysis

Commercial software (SPSS for Windows Release 25.0 Program, SPSS Inc, Chicago, IL, USA) was used to conduct statistical analyses. All of the data were visually examined, the Shapiro-Wilk test was used to check for normality, and descriptive statistics were conducted. Every set of data that passes the normality test was shown with its mean and standard deviation. The variables that did not pass the normality test are indicated with a "#" and displayed as the median and interquartile range (IQR). Independent Samples t test (alternative; Mann-Whitney U test) was used for comparisons between groups. In repeated measures, ANOVA was used to compare between measurements. The Bonferroni and Tukey HSD tests were used in post hoc comparisons. The relationship between categorical variables was evaluated using Pearson's χ^2 test (and Fisher's exact test). For every analysis, a P-value of less than 0.05 was considered statistically significant.

Results

During the study period, a total of 60 dogs brought to Erciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine, Small Animal Clinic were diagnosed with canine parvoviral enteritis. Thirty-seven out of the sixty dogs diagnosed with parvoviral enteritis met the inclusion criteria for the study. Of these sixty dogs with CPE, 23 were excluded from the study because they did not meet the inclusion criteria.

In the ST+Filg group, 19 owned dogs with parvoviral enteritis, consisting of various breeds were included. Among these 19 dogs, 11 (57.9%) were male, and 8 (42.1%) were female. The average age of these dogs was 136 (80-155) days. The dogs included in the ST+Filg group were of various breeds, including Akbash (n=1), German Hunting Terrier (n=1), Belgian Malinois (n=2), Border Collie (n=1), Doberman Pinscher (n=2), Kangal Shepherd Dog (n=3), Crossbreed (n=3), Pointer (n=1), Bolonka (n=3), Siberian Husky (n=1), and Terrier (n=1). Their median body weight was 9.50 (5.50-17.75) kg.

In the ST group, 18 owned dogs with parvoviral enteritis, consisting of various breeds were included. Among these 18 dogs, 10 (55.6%) were male, and 8 (44.4%) were female. The median age of these dogs were 79 (69-120) days. The dogs included in the ST group were of various breeds, including German Shepherd Dog (n=4), Belgian Groenendael (n=1), Akbash (n=2), Doberman Pinscher (n=1), Kangal Shepherd Dog (n=4), Crossbreed (n=2), Rottweiler (n=3), and Bolonka (n=1). Their median body weight was 6.90 (4.23-10.0) kg.

Vaccine and Antiparasitic Drug History Information

According to the history obtained from the pet owners, it was determined that out of these 37 dogs, 17 had received both internal and external parasite treatments regularly and on time, 8 had not received any, and 11 had an unknown anti-parasitic treatment status. Among these dogs, 15 had received a single combined (canine distemper, canine infectious hepatitis, canine infectious laryngotracheitis, canine parvovirus, canine parainfluenza, and canine leptospirosis) vaccine, and 11 had received twice the combined vaccines. The vaccination status of the rest of the 11 dogs were unknown.

Feces Antigen Testing

In cases of suspected canine parvoviral enteritis based on clinical examination, 37 dogs were evaluated as CPV antigen positive in analysis with lateral flow immunochromatography (LFI) rapid diagnostic kits for CPV antigen. PCR was also performed on the same fecal samples. Following the use of specific primers, PCR amplification was carried out on 1.7% agarose gel, and the results were evaluated under UV light. The samples from all dogs were evaluated as positive by PCR as well. Only 7 samples of PCR images are shown *(Fig. 2)*.

Physical Examination Findings

In the clinical examination of these 37 dogs with CPE in the ST + Filg (n=19) and ST (n=18) groups when they were admitted to the hospital, diarrhea (haemorrhagic diarrhea=22, non-haemorrhagic diarrhea=15) in 100% (37/37), vomiting in 86.49% (32/37), anorexia in 86.49% (32/37), lethargy in 78.38% (29/37), depression in 94.59% (35/37), dehydration (mild=7, moderate=9, severe=18) in 91.89% (34/37), enlargement of retropharyngeal lymph nodes in 75.68% (28/37), poor pulse quality in 59.46% (22/37), tachycardia in 62.16% (23/37) and fever in 43.24% (16/37) were detected.

The mean/median values of body temperature (°C), heart rate (bpm), and respiratory rate (breaths/min) for the dogs with CPE in the ST+Filg group when they were admitted to the hospital were measured as follows: 38.9 (38.1-39.9), 131.61±34.82, 32.00 (29.50-45.00), respectively. There was a statistically significant difference between the body

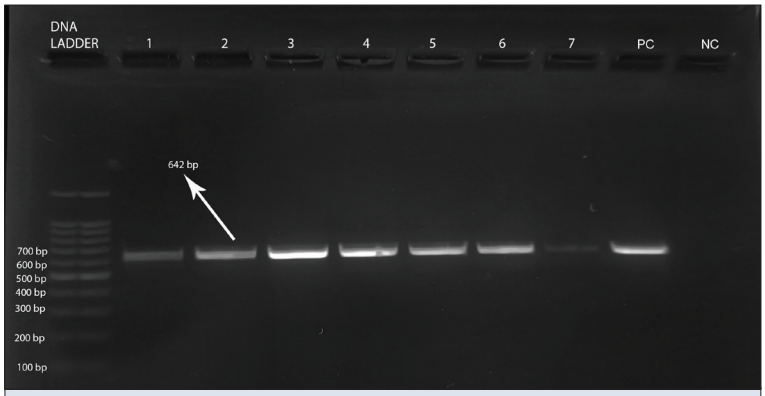

Fig 2. *Canine parvovirus* VP2 gene PCR results. Lines 1-7 represent the samples, where PC stands for the positive control and NK stands for the negative control (nuclease-free water). The specific primers used targeted a 642 bp PCR amplicon length, and all samples, including PC (positive control) and samples 1-7, were evaluated as positive

temperature measurements of dogs in the ST+Filg group (P<0.001). The mean body temperature on the 3rd and 5th days after treatment was significantly lower than on the 0th day (P=0.001, P=0.023). However, there was no statistically significant difference in respiratory rate and heart rate measurements of dogs in the ST+Filg group. The mean/median values of body temperature (°C), heart rate (bpm), and respiratory rate (breaths/min) for the dogs with CPE in the ST group when they were admitted to the hospital were measured as follows: 38.2 (38.0-38.7), 168.88±11.74, 30.00 (25.0-35.0), respectively. There was no significant difference between body temperature (°C), heart rate (bpm), and respiratory rate (breaths/min) measurements of dogs in the ST group (*Table 1*).

The mean CRT (sec) values of dogs with CPE in the ST+Filg group on the 3rd (2.24±0.75 sec) and 5th days (2.41±0.80 sec) after treatment were found to be significantly lower than the mean CRT (3.41±0.87 sec) values obtained on day 0 (P=0.001, P=0.002; respectively). There was no significant difference between the 3rd and 5th days in the ST + Filg group in terms of the CRT variable (P = 0.188). The average CRT (2.24±0.75 sec) values of dogs in the ST+Filg group on the 3rd day was found to be significantly lower than the same day measurement (3.29±0.29 sec) of the ST group (P=0.002) *(Table 1)*.

In the ST+Filg group, it was determined that 16 out of the 19 dogs treated with filgrastim (r-metHuG-CSF) (84.2%) showed improvement, while three of them (15.8%) did not survive. In the ST group, deaths were higher and most of the dogs died within the first 5 days. Of the 18 dogs included in the ST group, 12 recovered and 8 died *(Table 1)*. There was no statistically significant relationship between survival status and group categories of dogs with CPE in the study (χ^2=3.633, P=0.060). Leukocyte and neutrophil values of dogs with CPE that died were lower than those that survived (P<0.05) *(Table 1)*.

Blood Pressure Measurement Findings

The median SBP, DBP and MAP values of the dogs with parvoviral enteritis included in the ST+Filg and ST group when they were brought to the hospital were at the upper limit of the reference ranges specified for dogs [28]. There was no significant difference between the blood pressure measurements (SBP, DBP, MAP) of dogs with parvoviral enteritis in the ST+Filg and ST groups on the 3rd and 5th

Table 1. *Comparison of physical examination findings and survival status in dogs with parvoviral enteritis*

Variables		0th day (n=19)	3rd days (n=16)	5th days (n=16)	P Values	Ref. Ranges [26,27]
T (°C)#	ST + Filg.	38.9 (38.1-39.9)^{a}	38.2 (37.3-39.0)^{b}	38.4 (38.2-38.8)^{ab}	**<0.001**	37.5-39.2
	ST	38.2 (38.0-38.7)	38.2 (38.1-38.9)	38.6 (38.1-39.0)	0.574	
	P values	0.353	0.933	0.854		
RR (min)#	ST + Filg.	32.00 (29.50-45.00)	34.00 (26.00-44.00)	44.00 (30.00-52.00)	0.075	18.0-34.0
	ST	30.00 (25.00-35.00)	38.00 (29.00-51.00)	39.00 (32.00-51.00)	0.173	
	P values	0.990	0.116	0.320		
HR (bpm)	ST + Filg.	131.61±34.32^{A}	110.00±14.14	124.00±34.13	0.275	70.0-120.0
	ST	168.88±11.74^{B}	159.00±11.76	151.00±14.63	0.335	
	P values	**0.007**	0.059	0.867		
CRT (sec)	ST + Filg.	3.41±0.87^{a}	2.24±0.75^{b,A}	2.41±0.80^{b}	**<0.001**	<3.0 sec
	ST	3.79±0.42	3.29±0.29^{B}	2.86±0.34	0.065	
	P values	0.761	**0.002**	0.581		
Survival Status*						
Dead	ST + Filg.	0.0% (0/19)	15.79% (3/19)	15.79% (3/19)	χ^2=0.060 3.633	
	ST	0.0% (0/18)	33.33% (6/18)	44.44% (8/18)		
Survived	ST + Filg	100% (19/19)	84.21% (16/19)	84.21% (16/19)		
	ST	0.0% (0/18)	66.66% (12/18)	55.55% (10/18)		

*ST: Standard treatment, ST+Filg.: Standard Treatment + Filgrastim, CRT: Capillary refill time, HR: heart rate, T: body temperature, RT: Respiration rate. For each set of data that passes the normality test, the mean and standard deviation (SD) are displayed. The variables designated with a # and displayed as the median (25th-75th percentile) are those that did not pass the normality test. *The data were expressed as % (n /total). ^{a,b} Values within a row with different superscripts differ significantly at P<0.05*

Table 2. *Comparison of blood pressure before (day 0) and after (days 3 and 5) treatment*

Variables		0th day (n=19)	3rd days (n=16)	5th days (n=16)	*P* values	Ref. Ranges [28]
SBP (mmHg)	ST + Filg.	133.00 (101.00-139.00)^{A}	125.00 (114.75-128.50)	120.50 (109.00-126.50)	0.207	90-140 mmHg
	ST	128.00 (114.75-156.75)^{B}	118.00 (108.75-139.50)	128.00 (118.50-154.75)	0.639	
	P values	**0.045**	0.429	0.102		
DBP (mmHg)	ST + Filg.	125.00 (114.75-128.50)^{a}	80.00 (70.00-93.25)^{b}	77.50 (67.50-101.75)^{b}	**<0.001**	50-80 mmHg
	ST	91.50 (78.25-104.50)^{a}	74.00 (62.00-95.50)^{b}	101.00 (91.25-104.00)^{b}	**0.004**	
	P values	0.580	0.755	0.095		
MAP (mmHg)	ST + Filg.	90.00 (77.00-107.00)	96.00 (86.25-106.75)	91.00 (85.00-103.50)	0.220	60-100 mmHg
	ST	101.00 (92.50-119.75)	95.50 (81.25-139.25)	110.00 (99.00-120.25)	0.843	
	P values	0.141	0.495	0.150		

ST: Standard Treatment, ST+Filg.: Standard Treatment + Filgrastim, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure. Data were espresse as the median (25th-75th percentile). ^{a,b} *Values within a row with different superscripts differ significantly at P<0.05*

Table 3. *Comparison of hematological parameters before (day 0) and after (days 3 and 5) treatment*

Variables		0th day	3rd days	5th days	P Values	Ref. Ranges [29,30]
WBC (10^9/L) #	ST + Filg.	1.90 (1.30-4.95)^{a}	6.60 (5.10-16.10)^{ab,A}	13.40 (11.78-18.33)^{b,A}	**0.013**	6.00-17.00
	ST	2.75 (1.00-4.30)^{a}	3.30 (3.00-5.10)^{b,B}	7.98 (2.30-12.90)^{c,B}	**<0.001**	
	P Values	0.654	**0.004**	**0.005**		
Lymph (10^9/L) #	ST + Filg.	0.80 (0.35-1.15)^{a}	2.20 (1.40-2.80)^{b,A}	3.20 (2.05-5.13)^{b,A}	**0.017**	0.90-5.00
	ST	0.86 (0.40-1.10)^{a}	1.20 (0.60-1.59)^{b,B}	2.14 (2.10-5.10)^{c,B}	**<0.001**	
	P Values	0.918	**0.007**	**0.041**		
Mono (10^9/L) #	ST + Filg.	0.20 (0.20-0.40)	0.70 (0.40-1.20)^{A}	1.15 (0.90-2.35)	0.112	0.30-1.50
	ST	0.27 (0.18-0.38)^{a}	0.44 (0.20-0.54)^{b,B}	0.56 (0.30-0.80)^{b}	**0.001**	
	P Values	0.975	**0.042**	0.052		
Neut (10^9/L) #	ST + Filg.	1.00 (0.50-3.35)^{a}	4.00 (2.30-12.70)^{ab,A}	9.40 (6.00-13.25)^{b,A}	**0.007**	3.50-12.00
	ST	1.61 (0.50-3.10)^{a}	2.50 (2.00-3.22)^{b,B}	5.06 (3.18-6.10)^{c,B}	**0.001**	
	P Values	0.557	**0.008**	**0.005**		
Eos (%) #	ST + Filg.	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.996	1.00-18.00
	ST	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.992	
	P Values	0.990	0.996	0.980		
RBC (10^{12}/L)	ST + Filg.	6.92±1.13^{a,A}	6.18±0.88^{b}	5.56±0.62^{b,A}	**0.004**	5.50-8.50
	ST	6.07±0.59^{a,B}	5.91±0.48^{a}	5.69±0.55^{b,B}	**<0.001**	
	P Values	**0.002**	0.152	**0.026**		
Hgb (g/dL)	ST + Filg.	14.38±3.03^{a,A}	12.46±2.91^{b,A}	10.17±1.74^{b}	**0.004**	12.00-18.00
	ST	11.40±1.70^{a,B}	10.80±1.01^{a,B}	9.84±1.56^{b}	**0.002**	
	P Values	**<0.001**	**0.045**	0.064		
Hct (%)	ST + Filg.	40.92±8.69^{a,A}	35.20±7.73^{ab,A}	29.03±3.84^{b}	**0.002**	37.00-55.00
	ST	34.06±4.41^{a,B}	32.84±2.95^{a,B}	28.26±2.85^{b}	**<0.001**	
	P Values	**<0.001**	**0.044**	0.069		
MCV (fL)	ST + Filg.	57.96±7.69	56.63±8.44	53.30±4.70	0.203	60.00-72.00
	ST	55.69±4.96	55.19±4.39	53.67±2.89	0.240	
	P Values	0.301	0.467	0.926		

MCH (pg)	ST + Filg.	20.36±2.50[A]	20.06±3.23	18.30±2.42	0.276	19.50-25.50
	ST	18.57±1.39[B]	18.24±0.87	18.87±1.44	0.365	
	P Values	**0.008**	0.088	0.728		
MCHC (g/dL)	ST + Filg.	35.23±1.49	35.40±1.25	34.90±1.86	0.651	32.00-38.50
	ST	33.55±2.58	33.24±1.84	35.33±1.23	0.419	
	P Values	0.056	0.068	0.554		
RDWa (fl)	ST + Filg.	49.36±3.09	48.00±3.12	49.50±5.46	0.276	35.00-65.00
	ST	48.90±1.92	47.08±3.01	46.90±3.63	0.202	
	P Values	0.638	0.100	0.358		
RDW (%)[#]	ST + Filg.	15.40 (14.55-19.70)	15.10 (14.90-20.10)	18.10 (15.50-24.83)	0.449	12.00-17.50
	ST	17.21 (14.80-21.80)	17.54 (15.10-21.40)	17.11 (14.60-24.50)	0.176	
	P Values	0.592	0.929	0.494		
PLT (10^9/L)	ST + Filg.	301.18±91.39	297.57±170.70	261.00±91.33	0.156	200.00-500.00
	ST	366.10±182.52[a]	263.70±133.00[a]	180.02±44.94[b]	**0.005**	
	P Values	0.273	0.884	0.449		
MPV (fL)	ST + Filg.	6.45 ± 0.66	6.13 ± 0.66	6.40±1.41	0.513	5.50-10.50
	ST	6.61±0.98[a]	5.93±0.54[b]	6.04±0.41[b]	**0.022**	
	P Values	0.706	0.667	0.724		

ST: Standard treatment, ST+Filg.: Standard Treatment+Filgrastim, RBC: Red Blood Cell, Hct: hematocrit, Hgb: hemoglobin concentration, Lymph: lymphocyte, Neut: Neutrophil, Mono: monocyte, Eos: Eosinophil, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin volume, MCHC: mean corpuscular hemoglobin concentration, WBC: White Blood Cell, PLT: platelet, RDW: Red cell distribution, RDWa: absolute value of the width of the distribution of red blood cells, MPV: mean platelet volume. For each set of data that passes the normality test, the mean and standard deviation (SD) are displayed. The variables designated with a [#] and displayed as the median (25th-75th percentile) are those that did not pass the normality test. [a,b,c] Values within a row with different superscripts differ significantly at P<0.05

days. DBP values of dogs with parvoviral enteritis in the ST+Filg and ST groupS on days 3 and 5 were found to be significantly lower than day 0 (P<0.05) *(Table 2)*.

Hematological Findings

The mean WBC, lymphocyte, monocyte and neutrophil values of dogs in both groups (ST+Filg and ST) were significantly lower than the reference ranges determined for dogs at time 0 *(Table 3)*. In the ST + Filg group, there was a significant difference between pre-treatment (0th day) and post-treatment measurements (3rd and 5th days) in terms of WBC, lymphocyte and neutrophil variables (P=0.006, P=0.004 and P=0.007, respectively. The median WBC value on the 5th day after treatment was significantly higher than on the 0th day (P=0.001). The median lymphocyte value on the 3rd and 5th days after treatment was significantly higher than on the 0th day (P=0.048, P=0.004, respectively). The median neutrophil value on the 5th day after treatment was significantly higher than on the 0th day (P=0.006) *(Table 3)*.

Median WBC, lymphocyte, monocyte and neutrophil values of dogs with parvoviral enteritis in the ST group on the 5th day after treatment were significantly higher than before treatment (0th day) (P<0.001, P<0.001, P=0.001, P<0.001, respectively). Median WBC, lymphocyte, monocyte and neutrophil values on the 3th day after treatment were significantly higher than on the 0rd day (P=0.005, P=0.023, P<0.001, P=0.004, respectively). Median WBC, lymphocyte and neutrophil values on the 5th day after treatment were significantly higher than on the 3rd day (P=0.006, P=0.005, P=0.031, respectively) *(Table 3)*.

The median WBC, lymphocyte and neutrophil values of the dogs in the ST+Filg group on the 3rd and 5th days were significantly higher than the same day measurements of the ST group (P<0.05) *(Table 3)*.

The measurements were taken before treatment (day 0) and after filgrastim (r-metHuG-CSF) therapy (days 3 and 5) in the ST+Filg group showed a statistically significant difference in the RBC, Hgb, and Hct variables (P=0.004, P=0.004, and P=0.002, respectively). Furthermore, the mean RBC value on the 3rd and 5th days after treatment was significantly lower than on the 0th day (P=0.016, P=0.001, recpectively). In addition, the mean Hgb value on the 3rd and 5th days after treatment was significantly lower than on the 0th day (P=0.011, P=0.002; recpectively) and also the mean Hct value on the 5th day after treatment was significantly lower than on the 0th day (P=0.001). Other hematological variables were not statistically significant *(Table 3)*.

In the ST group, the mean RBC, Hgb and Hct values on days 5 treatment was significantly lower than on day 3 (P<0.001, P=0.001, P<0.001, recpectively) and on day 0

(P<0.001, P=0.009, P<0.001, recpectively). The median Hgb and Hct values of the dogs in the ST+Filg group on the 3rd days were significantly higher than the same day measurements of the ST group (P=0.045, P=0.044, recpectively) *(Table 2)*. The median RBC value of the dogs in the ST+Filg group on the 5rd days were significantly lower than the same day measurements of the ST group (P=0.026). Other significant differences between groups and measurements are given in *Table 3*.

Discussion

Canine parvovirus (CPV) and canine distemper virus (CDV) are two important pathogens that can infect both domestic and wild animals [1,4,31-33]. Leukopenia and neutropenia in dogs with CPE are regarded as negative prognostic indicators [4,13,32,34]. Upon evaluating the data obtained from the present study, it was determined that out of these nineteen dogs treated with filgrastim [recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF)] in addition to standard treatment, sixteen of the CPV infected dogs (84.21%) showed improvement while the rest of three (15.8%) did not survive. Additionally, the blood parameters of WBC, lymphocyte, and neutrophil values significantly increased starting from the third day of filgrastim (r-metHuG-CSF) treatment.

Gülersoy et al.[17] in a study, concluded that in CPE only granulocytes were effected and filgrastim was effective on the granulocyte count in the groups they formed without taking into account leukopenia levels, but when their findings were examined, a decrease was observed in the Filgrastim group on days 1 and 3 compared to day 0. In the current study, dogs that did not show signs of leukopenia were not included to the study. Therefore, the effectiveness of Filgrastim (r-metHuG-CSF) on leukocytes was more clearly demonstrated.

Punia et al.[23] reported that all 11 dogs with hemorrhagic gastroenteritis treated with Filgrastim in addition to supportive treatment recovered. Similarly, all 31 CPE positive dogs treated with recombinant canine granulocyte-colony stimulating factor (rcG-CSF) were reported to recover [18]. In our study, the mortality rate on the 5th day was calculated as 44% (8/18) in the ST group receiving standard treatment. The higher mortality rate observed in this group compared to the ST+Flig group suggests that it may be related to the fact that the dogs in this group did not receive any drugs that increase leukocyte levels. The mortality rate (15.8%) in the ST+Filg group was lower than the ST group. This rate was found to be close to the mortality rate reported by Güleryüz et al.[17]. However, in the current study, dogs with significantly lower total leukocyte count and its subgroups were included in the study. Therefore, it was believed that present study was better designed to demonstrate the effectiveness of the drug. Several researchers have expressed that leukopenia and neutropenia are negative prognostic indicators in dogs with parvoviral enteritis [4,13,32,34]. Therefore, monitoring complete blood count parameters in dogs with parvoviral enteritis at the time of admission and post-treatment can provide valuable information for the prediction of prognosis. According to the literature, although it is stated that the success of treatment in dogs using dog-specific granulocyte-colony stimulating factor (rcG-CSF) is 100%, the important disadvantages of this agent should be taken into consideration since it is difficult to obtain from the market and its price is quite high.

In this study, it was observed that the median WBC, lymphocyte, monocyte and neutrophil values of the dogs included in the study were significantly lower than the reference values of healthy dogs [29]. In a study, in which the pathogenesis of canine parvoviral enteritis was investigated experimentally, four days after the virus inoculation, five of the eight infected dogs showed signs of lymphopenia; five days later, all of the infected dogs showed signs of lymphopenia. The mean total leukocyte of virus-infected dogs was lower five and six days after virus inoculation compared to dogs that were passively vaccinated and control dogs, although the total leukocyte counts of individual dogs varied greatly. Five days following the inoculation, one infected dog was panleukopenic [35]. A possible explanation for our findings may be related to the fact that dogs with CPE, included in the present study, were brought to the hospital at an advanced stage of the disease, that is, after at least 4 days had passed since the onset of the disease. Indeed, as described by Mazzaferro et al.[1] bone marrow depression and CPV's attack on the bone marrow, thymus, and other lymphoid tissues are observed to cause leukopenia, lymphopenia, and neutropenia in the advanced stage.

CPV infection is associated with non-specific symptoms such as pyrexia, lethargy, anorexia, and increased respiratory rate within 2-7 days after the onset of infection [35-37]. Gülersoy and Naseri [9] reported higher body temperature, heart, and respiratory rate values in dogs with CPE compared to healthy dogs. When the dogs with CPE were admitted to the current investigation, their mean body temperature was almost at the top limit of the reference values [26]. The CPE-positive dogs body temperature dropped on the 3rd and 5th days following treatment, and this drop was statistically significant (P<0.001) compared to the pre-treatment level. This could be connected to the dogs' improved general health upon admission and the way that sepsis or systemic inflammatory response syndrome (SIRS) cleared up after receiving medication [9]. The dogs in the present study were found to be dehydrated during a clinical examination conducted at the time of

their hospital admission, and their RBC, Hgb, and Hct levels were in close proximity to the upper limits of the reference values [29]. However, on the 3rd and 5th days after treatment, these values began to decrease. Actually, dogs with parvoviral enteritis experience considerable fluid and protein loss in their gastrointestinal tracts [38]. Because dogs with parvoviral enteritis that vomit and have diarrhea lose fluid, which causes hypovolemia and dehydration. These conditions impair tissue perfusion, which can cause changes in mucosal color, tachycardia, and delayed capillary refill time [1]. In our current study, the decrease in RBC, Hgb and Hct values may be related to the improvement of dehydration and general condition with fluid and electrolyte therapy, as well as the start of fluid intake and the decrease in diarrhea. Another possible reason could be related to anemia resulting from hemorrhagic gastroenteritis that occurs concurrently with diarrhea. Because, hemorrhagic gastroenteritis is a common clinical manifestation of CPE [1].

The median WBC, lymphocyte, and neutrophil counts were 1.90 (1.30-4.95), 0.80 (0.35-1.15) 10^9/L, and 1.00 (0.50-3.35) 10^9/L, respectively. To correct the leukopenia in these dogs, in addition to standard treatment, subcutaneous filgrastim (r-metHuG-CSF) was administered at a dose of 10 µg/kg once daily for 5 days. On the 3rd and 5th days after treatment, WBC, lymphocyte, and neutrophil counts increased to the normal reference range along with clinical improvement. These values were found to be significantly higher than the values on the 0th day. In addition, the median WBC, lymphocyte and neutrophil counts of dogs in the ST+Filg group on the 3rd and 5th days after treatment were found to be significantly higher than the same day measurements of dogs with ST group. Filgrastim (r-metHuG-CSF) primarily affects the bone marrow and stimulates the production of leukocytes. The vital molecules that regulate the differentiation, growth, and survival of blood cells are called granulocyte colony-stimulating factors, or G-CSF [16,18]. Additionally, a number of studies have shown that within 24 hours of dosing, G-CSF increases the generation and release of functional neutrophils in the bone marrow [17,39]. In contrast to the findings of Rewerts et al.[40] and Mischke et al.[41] the results obtained from the current study were consistent with the recent studies [17,23,24]. Punia et al.[23] administered a daily dose of 10 µg/kg of filgrastim for three days as supportive treatment to 11 dogs with severe leukopenia and neutropenia due to hemorrhagic gastroenteritis. They reported that within 48 h of the last treatment dose, the hematological profile improved, and all dogs showed rapid clinical improvement without any problems. On the contrary, Gülersoy et al.[17] did not report a significant increase in WBC, lymphocyte and granulocyte counts on the 1st and 3rd days in dogs with parvoviral enteritis treated with filgrastim in addition to standard treatment. This may be related to the inclusion of dogs with parvoviral enteritis in the study without considering their leukocyte levels. When the data in the study of Gülersoy et al.[17] is examined, lowest and highest values of the WBC, lymphocyte and granulocyte explain this situation. On the other hand, in dogs with parvoviral enteritis, impressive results have been reported with recombinant canine granulocyte-colony stimulating factor (rcG-CSF) by many other researchers as reported in the present study [16,18].

The main limitation of the present study is low sample size. The diversity of breed variation in dogs, especially the susceptibility of certain dog breeds to CPV, is a well-known factor [5]. Nevertheless, in this study, Filgrastim demonstrated no adverse effects, raised leukocyte levels to the target range from the third day, and proved to be more cost-effective than other medications.

In conclusion, this study found that filgrastim (r-metHuG-CSF) improved leukopenia in dogs with CPE in addition to aiding in their clinical recovery. The results imply that filgrastim, an immunostimulant medication, may be included in therapy regimens to raise leukocyte counts in the management of canine parvoviral enteritis.

Declarations

Availability of Data and Materials: The data given in this study may be obtained from the corresponding auther (G. Ekinci) on reasonable request.

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Ethical Approval: This study was approved by the committee of HADYEK- Local Ethics Committee for Animal Experiments office of Erciyes University with 07.09.2023/169 approval number.

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

Author Contributions: İK, MÇ, VG, AGG and ACO supervised the study. GE, ET, SK, ES and AMAA collected the data. GE and ET made the statistics. The first draft of the manuscript was written by GE, ET, SK and ES and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Development and Validation of a Solid Phase Radioimmunoassay System for the Determination of Pregnancy-Associated Glycoproteins in Bovine Serum

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Abstract

The Pregnancy-Associated Glycoproteins (PAGs) belong to aspartic protease family and are synthesized by trophoblast cells of ruminant placenta and secreted into maternal blood and milk. These biomarkers very closely associated to pregnancy have demonstrated their efficiency and reliability in detection of vital embryos or pregnancy loss. In order to measure the concentration of PAGs in bovine serum, a direct radioimmunoassay (RIA) system was developed and validated. The radioiodinated bPAG tracer was prepared by chloramine-T direct method and the solid phase was used in RIA/PAGs as a separation system. The optimization tests allowed to set the assays procedure as working buffers, time and temperature of the assays incubation. Assay sensitivity was 0.63 ng/mL, the intra and inter-assay CVs were 7.7% and 5.3% respectively and the accuracy of the assays was expressed by a good parallelism and recovery tests. The serums bPAG values determinated by this developed system were consistent with the reproduction status and pregnancy stages of cows. The developed RIA/PAG system can provide the laboratory with an efficient method for the accurate detection of pregnancy, which could offer veterinarians and farmers an important tool for the reproductive management in dairy herds. Moreover, this diagnostic tool can also be developed for other livestock species.

Keywords: Bovine pregnancy-associated glycoproteins, Radioimmunoassay, Serum, Solid phase, Validation

Introduction

Reproductive performances and efficiency in dairy herds are highly dependent on accurate and timely diagnosis of non-pregnant females [1,2]. Early detection of non-pregnant cows reduce the intervals between calving and consequently optimize milk production [3]. To identify pregnancy, among the most commonly used existing biomarkers [3,4], apart from conventional techniques [5-8], Pregnancy-Associated-Glycoproteins (PAG), since they were isolated and purified in ruminants, have revolutionized pregnancy monitoring. PAGs belong to the family of aspartic proteinases [9] and are synthesized by the trophoblast cells of the placenta of ruminant, then secreted into maternal blood circulation 20 to 22 days of gestation in goats and ewes, 30 days of gestation in cows and doe [4,10]. The presence of the proteins has also been determined in milk and enabled their use to detect embryonic and fetal mortalities [11,12]. PAGs concentrations in milk are 10 to 50 times lower than those in plasma [13] and 1.5% of the amount present in serum [14]. The quantification of the glycoproteins has been developed in different species [13], by immunological techniques, depending on the availability of equipement in each laboratory, such as RIA [15] and ELISA [14-16]. The PAG-RIA and PAG-ELISA tests had similar accuracies for the diagnosis of early pregnancy in dairy cows at day 28 after insemination [5]. Considering the economic impact of the quantification of PAGs on the management of reproduction in dairy farms, in-order to provide users with a diagnostic tool for pregnancy, to make the kits easily available in our country and to allow the expansion use of the technique, a direct, fast and reliable in-house RIA kit to measure PAGs has been developed. The present study describes the steps for preparing a basic reagents of PAG/RIA kit, as a purified radioiodinated bPAG tracer, solid-phase separation system and a range of standards, as well as the optimization of the assays and the analytical and clinical validation of the kit in bovine serum.

Material and Methods

Ethical Approval

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the work. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Reagents

Purified bPAG 1 (boPAG67kDa) and Rabbit Antiserum raised against caprine PAG 55+62 kDa (crude antiserum R#706) were purchased from Pr J.F Beckers and delivered by Bioproduct Consult SPRL (Belgium). ^{125}I as sodium iodide was supplied by Isotop (Hungary). Chloramine-T and Sodium-Metabisulphite were from Sigma Aldrich (Germany). All other reagents used in this study were of analytical reagent grade and were obtained from Fluka and Sigma Aldrich.

Preparation of Radioiodinated PAG Tracer

- Radioiodination of PAG Glycoproteins

Iodination of bovine PAG was carried out under a radiochemical hood, using the direct method of Hunter and Greenwood [17], already presented in a previous work [18] with some differences. Briefly, 10 µL of bPAG (10 µg) in phosphate buffer solution (0.1 M, pH:7.4) was mixed with 4 µL (688 µCi) of ^{125}INa. The Chloramine-T was added and stirred slightly for 1min at room temperature then the reaction was stopped by addition of Sodium Metabisulfite. 100 µL of potassium iodide (KI) are added to the mixture in order to facilitate the separation between the tracer and the free iodine during the purification step. The labeling activity was measured in an ISOMED activity meter (Capintec).

- Purification of the Tracer

The purification of the tracer was carried out by gel filtration on a glass column (33×1.5 cm) of Sephadex G-75, equilibrated and eluted with a phosphate buffer/BSA (0.1 M, pH 7.4, 1% BSA. 0.1% sodium azide). The radioactivity of an aliquot (10 µL), of each fraction collected at a flow rate of 1 mL/tube, was counted in NaI (Tl) scintillation counter (Perkin Elmer). The fractions corresponding to the peaks were pooled and evaluated for radiochemical purity (RC purity) and immunoreactivity. The obtained tracer was stored in aliquots of 150 to 200 µL, in the refrigerator (8°C) and in the freezer (-6°C).

- Evaluation of the Tracer

Radioiodination Yield: The radioiodination yield of the tracer ^{125}I-bPAG produced was evaluated by using the elution profile obtained by purification of the tracer. It represents in percent, the ratio of the activity associated with the molecule (µCi) and of the total activity (µCi).

Radiochemical Purity and Immunoreactivity: The radiochemical purity of the selected fractions was evaluated by paper electrophoresis and the specific and non-specific binding (NSB) binding rates were performed in duplicate. They were evaluated as presented previously [18].

Specific Activity: The specific activity of the tracer, estimated from the elution profile, was evaluated by the ratio of the activity of the tracer (µCi) and 10 µg of labeled PAG (assuming that the total amount of PAG used has been labeled)

- Stability of the Tracer

The stability of the tracers produced (^{125}I-bPAG$_{67KDa}$) was studied over a time (8 weeks) and a temperature of storage (-6°C and 8°C), by estimating the radiochemical purity (PRC%) and the specific binding (Bmax%), and non-specific binding (NSB%) of tracer.

Preparation of bPAG Standards

Bulk solution (1 µg/mL) of pure stock boPAG$_{67KDa}$ (lyophilized powder) was prepared by dissolution of 10 µg of boPAG in 10 mL of a young heifer serum (PAG-free serum) to minimize the matrix interferences, containing as conservator agent, sodium azide (<0.1%). Working standards, covering the concentration range from 0.25 ng/mL to 100 ng/mL were prepared. The standards were aliquoted (1 mL) in vials, then lyophilized and stored at 10°C. The boPAG$_{67KDa}$ working standards used in RIA assays were prepared by reconstitution of the lyophilized standards in nanopure water.

Purification of Antiserum and Preparation of Solid-phase

- Purification of Antiserum AntibPAG

Rabbit polyclonal antiserum PAG (Crude antiserum Anti-caPAG (55+62kDa): AS#706, PAG accession numbers P80935) was purified to obtain the globulin fraction by using the sodium sulphate precipitation method [19]. The antiPAG precipitate obtained was dissolved in a 0.01 M phosphate buffer, pH 7.4 then dialyzed overnight in a tubing (Spectra/Por Biotech), against a 0.005 M phosphate buffer pH 7.4. The obtained globulins after dialysis are stored in a glass flask, lyophilized, and stored at -6°C.

- Preparation of Solid Phase

Separation procedure used in the RIA assay was the solid phase system [20]. In the present assay, the purified rabbit PAG antibody was diluted to different titers (1/100-1/80000) and coated as procedure described previously [18]. The stability of coated tubes was studied over nine months

by estimating the specific binding rates (Bmax %) in titer of 1/40000. The tubes intended for non-specific binding (NSB) evaluation, were prepared by the same procedure, but the antibody was replaced by a buffer.

Optimization of Assays Parameters

The optimization of the developed kit was carried out by evaluating assay parameters related to assay procedure, such the assay buffer systems (phosphate and Tris HCl buffers), the incubation condition of assays (duration time: 1-24 h, temperature: 6°C, 22-25°C (room temperature) and 37°C and the number of washes of the assay tubes after decantation.

Radioimmunoassay Procedure of PAG

The assay involved 50 µL of standard/sample and 250 µL of tracer (25000-35000 cpm), added into in-house coated tubes (solid-phase system with 1/80000 antibody titer). The contents of the tubes were mixed and incubated overnight at room temperature, under shaking. Finally, the tubes were decanted, washed once with 500 µL/tube of washing solution and the radioactivity of each tube was measured for 1 min. the displacement curve of RIA inhibition standards has been plotted on the y-axis, in a linear scale of binding rates B/Bo ratio (ratio between the amounts of radiolabeled PAG bound to antibody, in presence and in the absence of unlabeled PAG) and on the x-axis, in a logarithmic decade of the standards PAG concentration.

Validation of PAG Radioimmunoassay

Several quality control parameters of the assays curves were calculated automatically by the LBIS software used with the LB gamma counter. it concerns the sensitivity of an assay, defined as the minimum detection limit (MDL) or the mean concentration minus three times the standard deviation of Bo and the expected concentrations at 80%, 50% and 20% of B/Bo intercepts. The reproductibility of the RIA assay was carried out using two serum samples taken from pregnant cows. It is determinated by calculating the intra- and the inter-assay coefficients of variation (CV%), respectively 8 times whithin the same assay and in five consecutive assays. The accurancy of the RIA test was assessed by parallelism, which was determined by evaluating the serial dilution of pregnant bovine serums containing high concentrations of PAG with PAG-free serum and by a recovery test, determinated by adding increasing concentrations of purified $boPAG_{67KDa}$ (5, 50 and 100 ng/mL) to bovine serum, containing known PAG concentrations (0.93 and 5.6 ng/mL). The calculated recovery rate was the percentage ratio between the observed and expected values (ng/mL). For the clinical validation of RIA assay, the serum of PAG concentrations was determinated in 17 dairy cows, including 5 non-pregnants cows and 12 cows at differents stages of gestation. Pregnants cows were monitored by veterinarian from insemination until confirmation of pregnancy by transrectal palpation. One blood sampling (10 mL) was taken from the jugular vein in dry tubes, the serum was separated by centrifugation at 3000 g for 15 min, and stored in freezer until assay. After one assay, the samples with higher PAG concentrations (>100 ng/mL) were diluted than re-assayed to approximate the true values. All assays were performed in duplicate.

To perform the clinical validation, the bPAG assay results determined by the developed kit had to be compared with those determined by a commercial kit, however, a problem in acquiring the commercial kits forced us to compare our results with the clinical informations and the events recorded by the veterinarian on the individual cow records during these inspections.

Data Analysis

Statistical analysis was performed by MS Excel and ANOVA test from XLStat statistical software. Statistical significance was considered at the $P<0.05$ level.

Results

Radioiodinated Tracer Quality

An example of the elution profile obtained by the purification of $^{125}I\text{-}bPAG_{67KDa}$ tracer was given in *Fig. 1*. It shows the presence of two peaks, eluted successively between fractions 16-19 and fractions 43-45. The first one with high activity corresponds to the tracer $^{125}I\text{-}bPAG$ and the second one represents the free iodine. The radioiodination yields of $^{125}I\text{-}bPAG$ determined from the elution profile was 80.76%. The specific activity of the radiolabeled tracer was 55.6 µCi/µg. The radiochemical purity was 97% and the maximum binding of labelled $boPAG_{67KDa}$ in presence of an excess of specific antibody was approximately 42.6%. The non-specific binding (NSB) was about 2%. The stability of the tracer $^{125}I\text{-}bPAG_{67KDa}$ recorded by radiochemical purety (RCP), showed

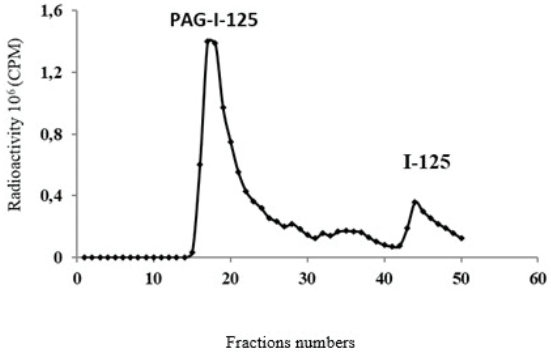

Fig 1. Pattern of elution of $^{125}I\text{-}bPAG_{67KDa}$

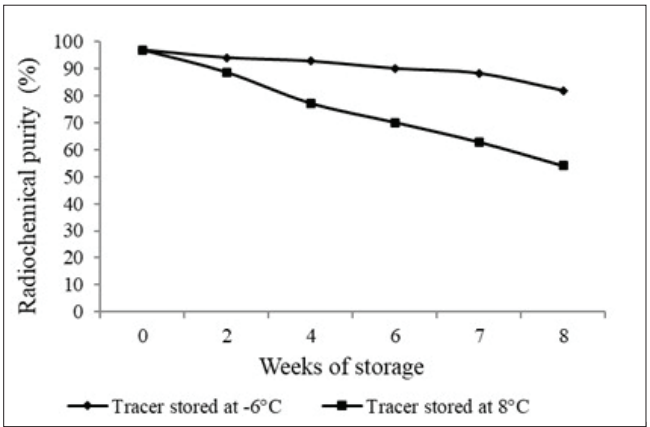

Fig 2. Stability of radiotracer monitored by radiochemical purity

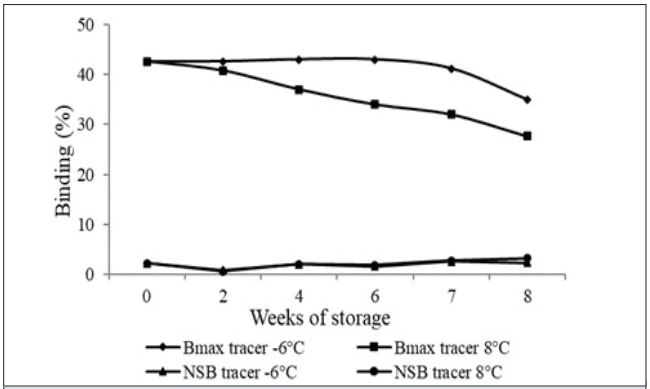

Fig 3. Stability of radiotracer monitored by immunoreactivity

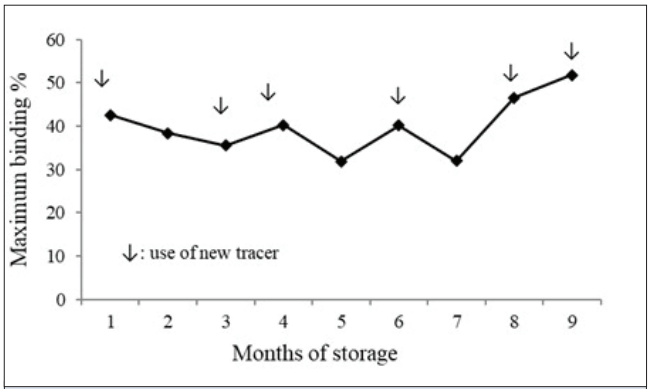

Fig 4. Stability of coating by immunoreactivity

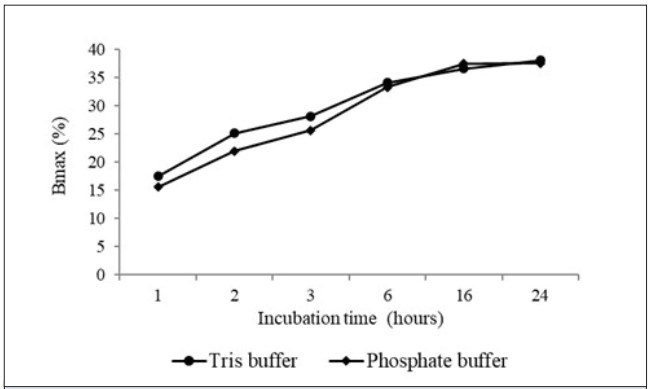

Fig 5. The effect of assay buffer and increasing incubation time on the level of specific binding

significant positif impact (P<0.05) of the storing tracer at a low temperature (-6°C) than 8°C *(Fig. 2)*, the means RCP rates obtained over entire storage period were 90.6±5.3% and 74.9±16.0% respectively. Concerning the storage time effect, the RCP values recorded for the two tracers (-6°C and 8°C), decreased very significantly (P<0.01) during the storage period, marking a stability between weeks 2-4 and 6-7. The degradation rate during the first month of storage was 4% and 20% respectively and it reached 16% and 44% throughout the storage period. The stability of the tracer recorded by specific and non-specific binding, significantly (P<0.01) confirms the interest of maintaining the tracer at a temperature of -6°C *(Fig. 3)*, the average values of Bmax obtained are 41.2±3.1% against 35.7±5.6% for the tracer stored at 8°C. As for the non-specific binding rates, the averages values recorded throughout the storage period of the two tracers (-6°C and 8°C) were respectively 1.9±0.6% and 2.1±0.9%. For the purposes of the RIA assays, the ^{125}I-bPAG$_{67KDa}$ tracer produced was pooled and stored at -6°C.

Solid Phase Quality

Stability assessment of the coated tubes, stored at a temperature of 4-8°C, performed over a period of nine months, showed no significant changes in immuno-reactivity results. The Bmax values obtained averaged 39.9±6.5%, varying from 32% to 52% *(Fig. 4)*, depending on the quality of tracers used to estimate the specific binding.

Assay Procedure Optimization

During the implementation phase of the assay, two buffer assay system were tested combined with gelatin (0.2%), the first one was the phosphate buffer (0.05 M, pH 7.4) and the second was Tris HCl buffer (0.025 M, pH 7.2). The specific binding levels recorded following the use of the two buffers systems were significantly (P>0.05) identical at all incubation period *(Fig. 5)*. The values obtained after 16 h of incubation of the assay are 37.5% for the phosphate buffer and 36.6% for the Tris HCl buffer. The same *Fig. 5* showed a very significant increase (P<0.001) in specific binding levels with increasing the assay incubation time from 16.6% (1 h) to 37.1% (16 h), reaching a plateau up to 37.9% (24 h). Regarding to the incubation temperature, the specific binding rates after 16 hours of incubation at temperature of 37°C, 22-25°C and 6°C, showed a significant difference (P<0.05) *(Fig. 6)*, the means rates recorded were 34.5±1.2%, 37.0±1.1% and 31.8±1.0% respectively. The shapes of the competition of kinetics reaction over all the incubation time of the 3 incubation temperature tested were identical. Finally, the assay tube washing step recorded no significant difference

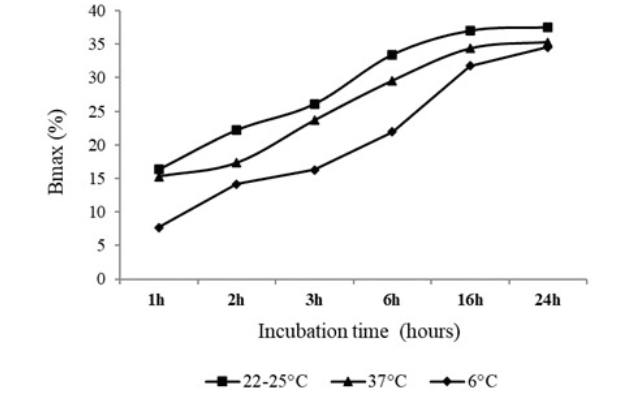

Fig 6. The effect of three incubation temperature on the level of specific binding

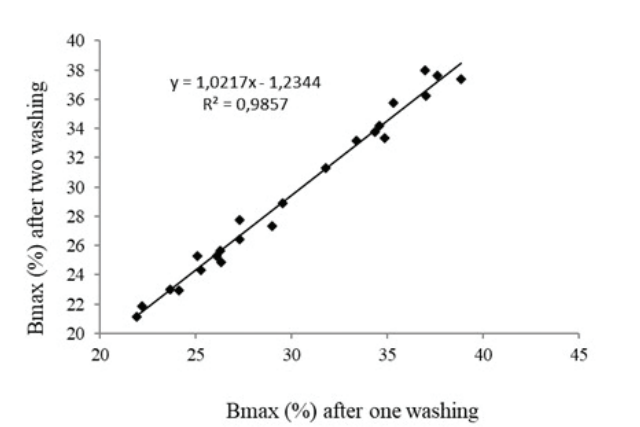

Fig 7. The effect of assay tubes washes on the level of specific binding

(P>0.05) in binding rates, when increasing tubes washes after decantation from one to two, the average rates recorded were respectively 29.9±4.8% and 29.1±5.0%. The maximum binding values obtained showed a good linear relationship between the two tested washes with a correlation coefficient R^2=0.99 and the regression equation of the following form y=1.02x-1.23 (n=23) *(Fig. 7)*.

Development of PAG Radioimmunoassay

A typical displacement of standard inhibition curve of the RIA/PAG system obtained was showed in *Fig. 8*. The mean standards inhibition curve ranged from 96.11% to 8.4% binding when 0.25-100 ng of boPAG was added per assay tube. The quality control parameters of standard curves tested over 10 assays *(Table 1)* showed no significant change (CV<10%). The mean maximum binding rate of 22.0±1.8% was obtained with ^{125}I-bPAG$_{67KDa}$ tracer and a final antibidy dilution of 1/80000. The nonspecific binding (NSB) value obtained was on average 2.4±0.2%. The developed RIA system can detect a wide range of PAG concentrations as shown by the expected PAG concentrations at 80%, 50% and 20% B/Bo intercepts, which were 1.5, 7.6 and 33.8 ng/mL respectively.

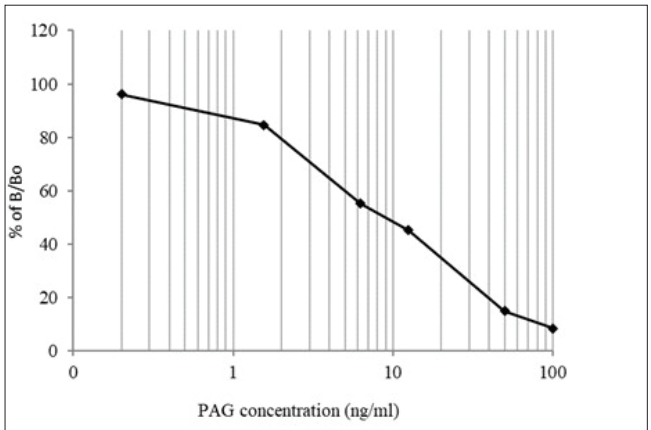

Fig 8. A typical standard curves for serum bPAG

Table 1. *Performances of quality control parameters of a standard curves*

Assay Parameters	**Results (n=10)**
Zero standard binding (%)	22.04±1.79
Non Specific Binding (%)	2.43±0.15
Expected Doses (ng/mL) at:	
20%B/Bo	33.83±1.66
50%B/Bo	7.61±1.26
80%B/Bo	1.54±0.43

Table 2. *Properties of the direct RIA kit for measuring pregnancy-associated glycoprotein in serum*

Parameter	**Value**
Sample volume (µL)	50
Measuring range (ng/mL)	0.25-100
Detection limit (ng/mL)	0.63
Dilution linearity (%)	104.3
Mean recovery rates (%)	
0.93 ng/mL	89.3
5.6 ng/mL	85.2
Intraassay CV (%)	
1.3 ng/mL	7.7
Interassay CV (%)	
26.3 ng/mL	5.3

Validation of PAG Radioimmunoassay

Validation properties of the direct RIA kit for measuring pregnancy-associated glycoprotein in serum was shown in *Table 2*. The minimal detection limit (MDL) of an assay was 0.63±0.20 ng/mL. The reproducibility of the RIA system (PAG_{706}) showed intra-assays and inter-assays CVs of 7.7% (1.3±0.1 ng/mL) and 5.3% (26.3±1.4 ng/mL) respectively. The accuracy of the assay was expressed by the good parallelism observed between the diluted bovine serum samples containing high concentrations of PAG and the curve of standards PAG (R^2=0.97, P<0.0009) *(Fig. 9)*

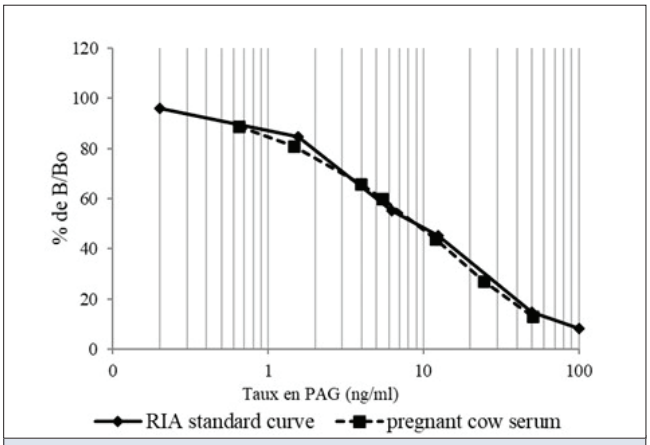

Fig 9. Parallelism between standard curve and serial dilutions of pregnant cow plasma sample.

Table 3. *Mean (±SD) PAGs serum concentration of non pregnant and pregnant recorded cows*

Physiological Status of Cows	PAG Concentration (ng/mL)
Non pregnant (n=5)	0.69±0.32
Pregnant (months)	
1 (n=1)	2.40
2 (n=3)	10.88±1.99
3 (n=1)	0.90
5 (n=1)	0.60
6 (n=1)	73.90
7 (n=2)	133.99±3.23
8 (n=1)	350.50
9 (n=2)	597.33±153.12

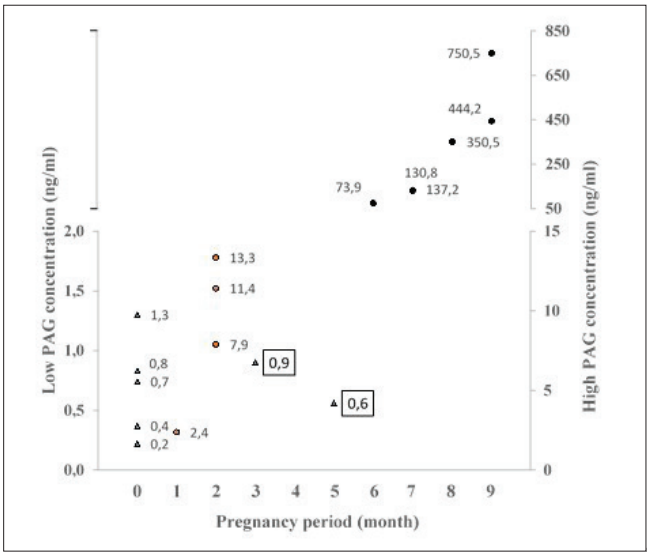

Fig 10. Serum PAG concentrations in various physiological status cows measured by in house RIA kit

and the satisfactory recovery of serial dilution of serum samples (104.3%) and mean recovery test of 89.3% (0.93 ng/mL) and 85.2% (5.6 ng/mL).,

The measurement of bPAG concentrations determined by the developed PAG_{706} RIA system, in serum samples from 5

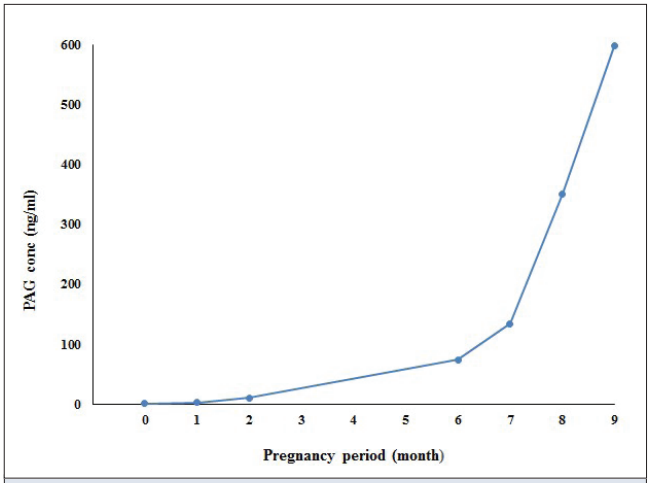

Fig 11. Pregnancy-associated glycoprotein (PAG) sera profile of tested cows during pregnancy

non-pregnant cows and 12 pregnant cows, showed values ranged from 0.22 to 750.45 ng/mL. For the non-pregnant cows, the average PAG concentration was of 0.69±0.32 ng/mL and for the pregnant cows, data revealed a constant increase of the PAG levels, depending on the month of pregnancy *(Table 3, Fig. 10)*. Two cows were sampled at the 3rd and 5th month of pregnancy, according to the veterinarian informations, recorded PAG concentrations of 0.9 and 0.6 ng/mL, respectively.

Discussion

Although the determination of the concentration of Pregnancy-Associated Glycoproteins (PAGs) has proven useful for monitoring pregnancy in cattle and other dairy animals over the past 20 years [21], the kits of assays are still not available in some countries which do not always have the means and the sufficient conditions to regularly provide kits. As part of the development study of reagents kits for in-house RIA determination of Pregnancy-Associated Glycoproteins (boPAGs) in bovine serum, the first basic reagent prepared was a radioiodine tracer. The assessment of the quality of the obtained tracer reveals very satisfactory results in terms of radiochemical purity and stability over time when it was stored at -6°C. The specific activity of the tracer was in the range of the values obtained previsouly (60-80 µCi/µg), from tracers radiolabeled with 1 mCi [22], it is directly related to the low dose of Na ^{125}I used for radiolabeling the proteins, compared to those previously reported (2 mCi) [15]. Maximal binding values of labelled $boPAG_{67KDa}$ were similar to those previsiouly reported [15,23], with a different RIA system and the non-specific binding (NSB) values were remained below the ideal limit of 5%.

Despite the widespread use of the immunoprecipitation separation system (double or second antibody precipitation) in all previously reported RIA/PAG studies [15,23-25], in

the RIA/PAG system developed, a solid phase separation system has been used and was has proven effective and stable over time. The advantage of the separation system is that it does not involves the development of a second antibody, a long incubation and a separation step by centrifugation, for these reasons, it was the most commonly used [26]. In addition, the separation system greatly minimizes the matrix effect [27].

The purpose of this step was to set the optimum conditions allowing the immunocompetition reaction to reach equilibrium. In the developed RIA/PAG, the phosphate buffer assay system was retained on Tris HCl buffer, which is most commonly used in later RIA/PAG assays [9,15,23-25]. A single incubation, at room temperature, for 16 h was necessary. In all previous studies, in the reported RIA/PAG systems developed, the optimal time and temperature for incubation of the validated assay reaction was 16 h at 22-25°C for the preincubation period, then 2 h at 20°C for the incubation period with added tracer [2,15,22-25,28-30]. Decanted tubes were washed only one time, reducing non-specific binding (NSB) to insignificant levels [31].

Direct assays with heterologous RIA systems, based on antisera raised against the caprine PAG ($caPAG_{55+62kDa}$) immunogen and the pure bovine $bPAG_{67KDa}$ used as standard and tracer have been undertaken. Compared to homologous RIA system, it was found to be more effective in detecting circulating PAG molecules [9,24]. The average maximum binding rate obtained was in agreement with previously reported specific binding values [9,15,24] and the non specific binding (NSB) value does not exceed the RIA limit of 5% [31]. The minimum detection limit value obtained by the developed direct RIA system was better (<0.8 ng/mL), than those determined with non-preincubated heterologous systems and standard curves ranged from 0.8 to 100 ng/mL [29] and it was in the range (0.3-0.7 ng/mL), of those determinated under the same conditions previously reported [24]. However, with the preincubated heterologous and homologous RIA systems and standard curves ranging from 0.2 to 25 ng/mL, except for the MDL value obtained by Barbato et al.[23], with RIA-706 (0.8 ng/mL), our obtained RIA sensitivity was lower (>0.4 ng/mL) than that previously reported [2,15,22,25,28-30]. The sensitivity of a test is related to the slope of the standard dose-response curve [31] and the use of non-equilibrium methods, which consist of mixing the antiserum and the sample, then after a predetermined time the tracer, has been found useful in some cases to increase the sensitivity of the assay [32]. The difference in the MDL values obtained both in the present study and in the previous ones [2,15,22-25,28-30] can be attributed to the assay procedure, to the solid-phase separation system used, or the calculation of the detection limit, which corresponds to the binding value of duplicate Bo minus 2SD(Bo) for the differents authors. Ayad et al.[33] attributed the differences in RIA assays sensibility to technical aspects, such as the iodination method, the dilution titres of the first antiserum or the incubation time after the addition of the second antibody precipitation system. Physiologically, the detection limit recorded with the developed RIA/PAGs is too close the threshold of 0.8 ng/mL, considered as the basic concentration in PAG assay (RIA-706) for the diagnosis of pregnancy in cows [24], 1.0 ng/mL for ewes [23], goats [2] and for buffalo cows [34]. The wide range concentrations that can be detected by the developed RIA system is sufficient to monitor PAG levels during the gestation of cows [24-29]. Additionally, the system may be very suitable for the determination of PAGs in sheep and goats sera [22,23]. Otherwise, according to Ciabattoni [35], the sensitivity of the assay can be improved by increasing the specific activity of the tracer, thanks to increase in the dose of radiolabeling activity to 1-2 mCi. The results of the analytical validation showed that the non preincubated heterologous RIA system developed (RIA-706) was precise and the accuracy very satisfactory. The performances recorded were comparable to those previously obtained with cows [5,15,33], with sheep [2], with goats [22], with zebu cattle [29] and with buffalo cows [23,30]. Clinically, the measurement of bPAG concentrations in serum samples from cows showed that the RIA-706 developed sytem was able to distinguish non-pregnant cows from pregnant cows and differentiate between each pregnancy stages. it also made it possible, in pregnant cow, after veterinary examination, to detect two cases of fetal mortalities. The PAG values obtained, according to the physiological status and the month of pregnancy of the sampled cows, are in agreement with those reported in previous studies [15,28].

A direct RIA assay to quantify PAGs in bovin sera was developed. The preparation and optimization steps of differents compoments of the kit were presented. Compared to previously reported RIA/PAGs [2,15,22-25,28-30,33], the separation system used was solid phase, requiring only one antibody, one incubation and non centrifugation. The resulting RIA assay was sensitive, reproductible, accurate and was able to distinguish among the serum samples analyzed, between the non-pregnant cows and pregnant ones and within the batch of pregnant cows, to detect cases of fetal mortality. In perspective, this study will be reinforced by another clinical validation, which will include a larger number of cows. the results obtained will be compared with those determined by commercial kit. it would also be wise to include in the study the synthesis of PAGs and the production of specific antibodies.

Declarations

Availability of Data and Materials: The datasets during and/or analyzed during the current study available from the corresponding

author (N. Mimoune) on reasonable request.

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Ethical Approval: All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Competing 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.

Author Contribution: AK, SB, AAA, RK: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. AK, RK, NM: Investigation, Writing - Review & Editing.

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Research Article

Regional Biophysical Variations in Canine Atopic Dermatitis: Non-Invasive Mapping of Skin Parameters

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Abstract

Mapping the skin with various biophysical properties can demonstrate not only skin condition but also organize a convenient approach to managing diseases. The aim of this study, mapping of the regional alteration for 6 biophysical parameters with non-invasive method. Totally 39 dogs [healthy (n=16) and canine atopic dermatitis (n=23)] were enrolled. Skin pH, hydration, melanin, sebum content and temperature for pinna, perilabial region, hind-fore feet (dorsal metacarpus-dorsal metatarsus), axillae, lateral thorax/flanks, elbows, abdomen/inguinal and ventral tail were measured. Atopic dogs exhibited more acidic skin (P<0.001) compared to healthy dogs, with the thorax/flanks region showing significant regional differences (P=0.037). Hydration levels were consistently lower in atopic dogs (P<0.001), with significant regional differences observed in the abdominal/inguinal region (P=0.037). Skin temperatures were generally higher in atopic dogs, with a significant increase noted in the tail (P=0.037), while other areas showed no significant differences. Melanin and sebum content did not exhibit significant regional variations. In conclusion, this study demonstrated regional variations in biophysical parameters in atopic dogs using non-invasive measurements. These findings provide valuable insights for understanding canine atopic dermatitis and its management.

Keywords: Atopic dog, Non-invasive measurement, Skin-barrier dysfunction, Skin hydration, Skin pH

Introduction

Canine atopic dermatitis (caD) is a chronic inflammatory, multicomplex allergic/pruritic skin disease [1,2] that is frequently encountered in dogs [3]. caD was aroused incidence/spatial distribution recognized for elevated skin permeability [4] and immune response [5,6]. Although the pathophysiology of caD is not fully understood, it was revealed that the skin barrier function is impaired, allergens infiltrate the skin stratum, and the immune system is activated leading to clinical symptoms [7,8].

Various biophysical parameters using noninvasive technics have been evaluated for human skin integrity [8-10]. In dogs skin integrity was mostly evaluated with transepidermal water loss (TEWL) [7,11,12]. Moreover, skin pH and hydration are alternative parameters for assessment [7,13]. A pilot study related to the comparison of skin barrier integrity with different methods among healthy and atopic dogs claimed that pH and skin hydration could be better biomarkers in contrast to TEWL [7].

As the largest organ of the body, the skin has most of its functions including protection by providing a physical barrier, sensation, heat regulation, and excretion in addition to the secretion that controlling water and electrolytes keeping [14]. Mapping of the skin with various biophysical properties can demonstrate not only skin condition but also organize a convenient approach to the management of diseases. In the present study, we aimed to investigate skin hydration, sebum content, melanin, temperature, and pH in the CADESI-4 specified body sites related to caD with mapping of skin.

Material and Methods

Ethical Statement

Dogs presented to the Animal Hospital of the Veterinary Faculty at Aydın Adnan Menderes University and diagnosed with atopic dermatitis were included in this study. Ethical approval was obtained from Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University (No: 64583101/2024/22). Additionally, comprehensive

Table 1. *Inclusion/exclusion criteria of the study*

Criteria	Healthy Dog	Atopic Dog	References
Inclusion criteria			
Anamnesis and clinical signs consistent with skin disease	-	at least one-year clinical history	Hensel et al.[16]
Favrot Criteria	-	at least 3 criteria	Favrot et al.[15]
Canine Atopic Dermatitis Extent and Severity Index (CADESI-04)	0	>10	Olivry et al.[1]
Exclusion criteria			
• At least two weeks treatment history with topical and/or systemic antibiotics, glucocorticoids, immunosuppressive and/or antipruritic therapy			
• To be used shampoo or cleaning solution in the last twenty-four hours			
• Concomitant endocrine disorders • Gastrointestinal disease (dehydration)			

information was provided to the owners of all dogs, and written consent was obtained from them.

Study Population: Exclusion and Inclusion Criteria

Different ages, breeds and both sexes, to those of dogs 16 healthy and 23 with atopic dermatitis were enrolled in this study.

The inclusion/exclusion criteria of healthy and atopic dogs were presented in *Table 1*. Dogs were composed of any history or clinical signs consistent with skin disease. Within the diagnosis of atopic dermatitis, studies by Favrot et al.[15] and Hensel et al.[16] as well as at least one year of clinical history were considered. The clinical index of atopic dogs was performed according to the canine atopic dermatitis extent and severity index (CADESI-4) [17].

Dogs were excluded if they had undergone at least two weeks of topical or systemic antibiotic, glucocorticoid, immunosuppressive, or antipruritic therapy, or if they had recently used shampoo or cleaning solution. Additionally, dogs with concurrent endocrine problems and gastrointestinal disease related dehydration were eliminated *(Table 1)*.

Clinical Scoring with PVAS and CADESI-4

Pruritis score was recorded using the pruritus visual analog scale (PVAS) as described previously by Hill et al.[18]. Briefly, The PVAS score was assessed by owners to indicating the severity of the itching on the scale that consists to 10 scales with 0 (no pruritus) to 10 (extremely pruritus).

CADESI-4 was used for evaluating skin lesion severity that were based on scaling of skin lesions (erythema, lichenification, and excoriation/alopecia) on each of 20 body parts with none to severe four-point and it was classified as mild (10 point), moderate (35 point) or severe (≥60 point) atopic dermatitis [1].

Biophysical Parameters

Before the beginning of measurement, all parameter cartridges were calibrated according to the manufacturer's instructions, and the calibration process was repeated in the same procedure when necessary. Hydration, sebum content, melanin, temperature, and pH parameters were measured at three times at the body sites specified CADESI-4 (hind-fore feet, axillae, perilabial region, pinna, ventral tail, lateral thorax/flanks, elbows, hindlimbs, abdomen/inguinal) by the same investigators and for each body sites scored according to Olivry et al.[1].

All parameters were assessed using the Corneometer (®Callegari, Soft Plus, Italy). With dogs in lateral recumbency/standing, the device probe was placed on both the right and left body side without the abdomen/inguinal region of the dogs. Measurements were obtained by placing the probes at the specified body sites. The working principle of all parameters was summarized in *Fig. 1*.

Skin hydration is based on a capacitive measurement principle of the dielectric constant that changed with the water of the skin. In addition to hydration, skin melanin measurement is based on strength of the absorbed and the reflected light at λ1=875 nm, λ2=660 nm. Skin surface temperature is based on non-contact infrared measurement.

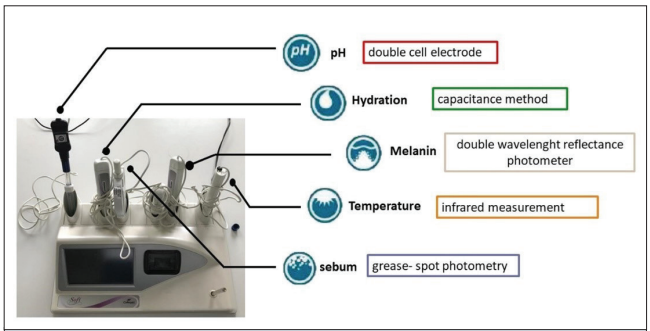

Fig 1. Working principle of all biophysical parameters

The skin surface pH was measured by a device probe that worked with ±1% accuracy based on double cell electrode system. The probe was removed from the buffer solution after it was kept at 24°C with 2 applications (a) activation button and b) sensor. Then, it was contacted with the skin by pushing it slightly forward with the activation button at the same time. Analysis and measurement were carried out in a very short time with a positive warning sound from the device.

Statistical Analysis

Statistical analyses were conducted using SPSS® Software version 26 (IBM SPSS Inc., Armonk, NY, USA). The normality distribution of continuous variables was assessed using the Shapiro-Wilk test, and data were presented as mean±standard error (SE). Mann-Whitney U Test was utilized for comparing groups and Spearman correlation were used for correlation analyses. A P-value of less than 0.05 was statistically significant.

Results

Demographic Data

Encountered total of 39 dogs were grouped as healthy controls (n=16) and caD (n=23). Out of dogs with caD, 18 were pure-bred and other relevant 5 mixed-breed. Pure-breed atopic dogs were consisted of French bulldog (n=4), Pomerian (n=4), Pug (n=3), West Highland white terrier (n=2), Yorkshire terrier (n=1), Golden retriever (n=1), Border collie (n=1), Dogo Argentino (n=1), Toy Poodle (n=1). The mean age was 3.9 years (range 18 month-11 years) of dogs with caD and 3.9 years (range 1-7 years) of healthy.

Regional Variation of the Biophysical Parameters

The overall results for the biophysical parameters were summarized in *Table 2* and the heatmap for the visualization of group differences was shown in *Fig. 2*.

The mean pH, hydration, melanin, sebum and temperature (°C) overall were 5.66±0.18, 27.60±1.44, 27.20±3.04, 16.92±1.77 and 34.11±0.46 in atopic dogs versus 7.04±0.04, 43.38±2.73, 12.50±0.56, 38.52±1.75 and 32.61±0.23 in healthy ones, respectively. Considering the total mean variable, all parameters of atopic dogs were found to be significant compared to healthy (P<0.001). It was revealed that skin pH was more acidic in atopic dogs (P<0.001). Additionally, reduced hydration (P<0.001) and sebum (P<0.001) but increased melanin levels (P<0.001) and temperature (P<0.001) were observed in atopic versus healthy dogs.

Hydration levels were consistently lower in atopic dogs compared to healthy ones at all sites (P<0.001), but the regional statistical difference was only obtained for the abdominal/inguinal region (P=0.037). Considering

Table 2. *Regional skin pH, hydration, melanin, sebum and temperature values of healthy and atopic dogs*

Region	pH		P Value	Hydration		P Value	Melanin		P Value	Sebum		P Value	Temperature		P Value
	Atopic	Control		Atopic	Control		Atopic	Control		Atopic	Control		Atopic	Control	
Pinna	5.47±0.33	6.43±0.17	0.857	25.0±4.5	53.31±6.8	0.111	22.39±5.6	12.62±3.4	0.403	19.7±4.0	21.87±5.1	0.638	33.7±1.3	32.68±0.6	0.538
Axillae	5.93±0.28	6.60±0.1	0.491	21.65±4.4	38.75±5.8	0.403	29.95±6.5	10.12±2.7	0.363	18.88±4	35.75±4.5	0.857	33.31±1.3	34.57±0.4	0.691
Dorsal Metacarpus	5.66±0.3	7.19±0.1	0.403	30.30±5.4	32.00±4.5	0.290	18.08±4.8	17.50±2.9	0.745	16.65 ±4.1	36.75±3.4	0.174	33.80±0.6	29.30±0.4	0.691
Dorsal Metatarsus	5.38±0.3	7.46±0.1	0.857	20.78±5.8	27.50±3.7	0.446	20.65±5.7	9.31±1.5	0.801	11.30 ±3.1	42.56±3.1	0.691	34.76±0.4	29.03±0.3	0.174
Abdominal/ Inguinal	5.88±0.3	7.44±0.1	0.446	24.73±5.9	68.31±6.7	0.037	32.3±7.2	8.87±1.6	0.914	15.95±4.2	43.75±3.1	0.446	33.83±0.4	33.1±0.7	0.801
Perilabial region	5.64±0.3	7.07±0.1	0.257	33.47±7.1	34.00±3.1	0.971	34.26± 7.8	19.43±2.7	0.257	15.30±3.5	43.75±3.7	0.587	34.49±0.3	33.23±0.7	0.080
Tail	5.92±0.3	7.12±0.1	0.691	25.08±5.3	25.00±3.8	0.055	35.86±6.4	12.43±2.9	0.691	18.30±4.3	37.00±3.3	0.801	32.55±1.6	32.4±0.6	0.037
Elbow	5.06±0.2	6.97±0.1	0.403	29.8±7.6	42.87±5.1	0.587	26.39±7.0	12.37±2.8	1.00	19.73±4.2	40.81±4.1	0.638	35.22±0.5	34.13±0.4	0.363
Thorax/ Flanks	5.90±0.2	70.7±0	0.037	37.47±7.9	68.75±5.6	0.325	24.86±4.8	9.87±2.3	0.587	16.30±4.4	44.50±4.0	0.691	35.17±0.3	35.00±0.4	0.691

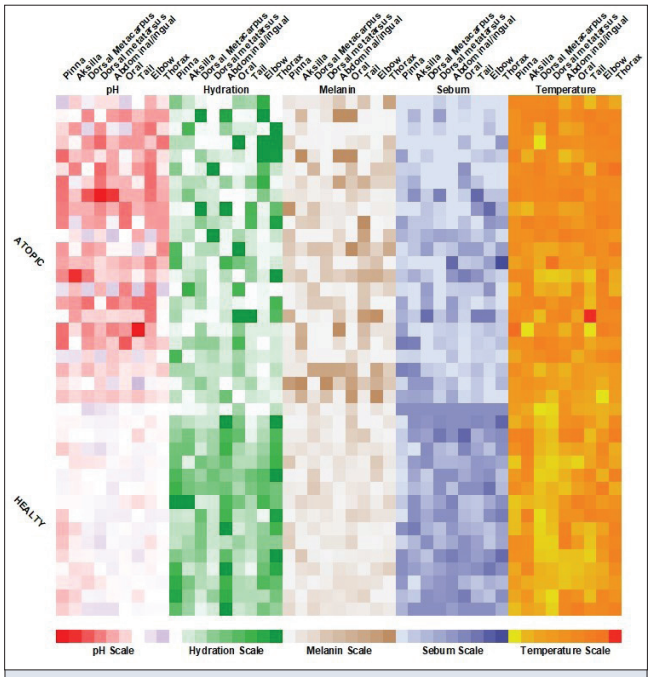

Fig 2. Heatmap colour visualization providing to the regional alteration of the biophysical parameters by compared group

the regional distribution of pH in atopic dogs, it was seemingly more acidic for all the regions (P<0.001), however, a significant difference was only obtained for the thorax/flanks with lower than compared to healthy dogs (P=0.037). Although there was a statistical difference for the mean temperature of atopic dogs as like others (P<0.001), considering regional distribution, it was only higher for the tail with 32.55±1.6 versus 32.4±0.6 at the healthy (P=0.037). There was no significant regional variation of melanin and sebum content in comparison to the healthy group (P<0.05) *(Table 2)*.

Clinical Scoring

Overall, moderate to severe clinical signs were observed in the caD group according to CADESI-4 with the mean score measured as 49.4 (11-180) with mild (n=18) to severe (n=5). Additionally, the mean PVAS was obtained at 7.6 (4-10). For the healthy group, CADESI-4 and PVAS scores were obtained as 0. No correlation (P>0.05) was found between biophysical parameters and the CADESI-4 severity or PVAS in a selected region of atopic dogs.

Discussion

Canine atopic dermatitis is a complicated disease originated from interactive relation between multiple factors mostly related of allergy. All of factors induce caD with disrupting the skin barrier function and leading immunological response. There is increasing evidence, primarily in humans but more recently in dogs, indicating that an impaired skin barrier plays a significant role in the pathophysiology of atopic dermatitis [7,19,20]. There are many clinical and immunological similarities including the distribution of clinical lesions and exposure to allergens through the skin between humans and dogs that ensured the encouragement to research of skin barrier distribution in caD [21]. For this purpose, we aimed to presented various skin biophysical properties including hydration, sebum content, melanin, temperature, and pH to being CADESI-4 specified body sites of dogs with atopic dermatitis for evaluation of skin barrier integrity. We indicated to alteration in the biophysical properties of caD with mapping.

Skin barrier function with biophysical properties of atopic dogs have been investigated by a few studies [7,22,23]. Zając et al.[22] was observed that skin erythema intensity, hydration, and pH might be used for the evaluation of skin lesion severity, although the correlation is limited. In a more recent study performed by Cobiella et al.[7], they compared three consecutive measurements of skin hydration, pH, TEWL, skin absorbance and erythema in the pinna, axillae, interdigital and inguinal region, and they observed that those parameters were used for evaluating skin barrier dysfunction. Conversely, in the study essentially aimed to treatment efficiency of probiotic containing spray on clinical assessment and skin microbiota, they evaluated to skin barrier function. They found to hydration for pinna was reduced after 28 days treatment [23]. In our data was shown that although mean pH, hydration and sebum were downward tendency at all sites (P<0.001), regional variation was not the same for all parameters. The hydration for abdominal/inguinal, pH for thorax/flanks and temperature for tail were differently significant in caD compared with health dogs (P<0.05), however, there was no importance at regional values of sebum and melanin. In line with the investigations, our research suggests that the primary non-invasive parameters that serve as indicators of skin barrier dysfunction are skin pH, hydration, and temperature.

Disrupted skin integrity is having a role on the pathophysiology of caD [7,21]. Skin barrier homeostasis is mostly closely related to the network of microbiota, immune, and chemical stimulant [25]. In human atopy, pH is known to play a regulatory role in this communication [26]. caD was mostly found to be associated with increased skin pH [7,27] due to filaggrin dysregulation and bacterial dysbiosis [28]. Controversially, it has been stated that there are regional differences, as well [7]. In cited study, pH was measured usually higher in atopic dogs for only 4 body sites (inguinal, axillae, pinna and interdigital), however difference was found only for axillae and the inguinal region (P<0.05). Moreover, Oh and Oh [29] was reported to skin pH generally increased in 14 body sites without footpad, interdigital areas, and ears of healthy dog before

and after anesthesia. In our study skin pH was totally measured as acidic of atopic dogs compared to healthy (P<0.001) and only had statistical significance for thorax/flanks (P=0.037). Similar to our study, it is stated that the relationship between the decrease in pH and the high abundance of *S. aureus* was influenced in a physiological, microbiome, and clinical outcomes conducted on cases with atopic dermatitis [30]. Also, there are variable functional discrepancy between species including dermal cells and glands, enzymes or microbiome for maintaining optimal skin function [21,31]. Epidermal gland type [32,33], hairless [29], lesion [7,29] and enzyme secretion [21] play role on the pH regulation as cited by previous studies. Breed, age, time, excitement as other factors also have to be affected on skin pH [34,35].

Lower skin hydration and TEWL were caused by the preventing of water flowing to the epidermis, otherwise increased TEWL and decreased skin hydration resulted from epidermal dehydration [36]. In both scenarios, reducing stratum corneum hydration results in dryness of skin, and dry skin has been linked to pruritus [37]. So, it is seen that the high TEWL and low hydration state has gained in characteristic features for atopic lesions [38,39]. Although a decrease in skin hydration and lower TEWL have traditionally been regarded as markers of skin integrity [36], Cobiella et al.[7] discovered that skin pH and hydration were superior biomarkers. Decreased skin hydration demonstrates to loss of cutaneous water capacitance that is not desired for physiological activity including epidermal cell proliferation and differentiation [40]. We observed more dehydrated skin compared with healthy dogs (P<0.001). Also, the abdominal/inguinal region indicated lower hydration compared to healthy dogs (P=0.037). This finding was compatible with a forementioned previous studies. It has been stated that TEWL decreases in different animal species such as horses [41], cats [42], and dogs. There are even statistical differences between dog breeds [34,43,44]. In our study, regional water loss was examined with skin hydration in 16 dogs with atopic dermatitis of different breeds. The effect of dog breed could not be investigated due to the small number of animals.

Skin water loss is controlled by passive diffusion through the epidermis, but at high temperatures, this transition is greatly affected by sweat penetrating and diffusing into the epidermis in mammalians [45]. Body temperature and age affect skin hydration and pH in humans. These parameters are also influenced by current skin temperature, blood flow and immunity, as well as ambient temperature [46]. Indeed, skin temperature and TEWL are closely related to the stimulation of sweat glands for secretion [47]. The skin temperature in dogs is generally ranged between 34.5 and 35.5°C [48]. In the present study, the mean skin temperature was measured as 34.11±0.46 in atopic dogs and it was only higher for tail compared to the healthy group (P=0.037). According to Breathnach et al.[44] there were no differences in mean skin temperatures between dogs with pododermatitis and healthy dogs. Our findings indicate that while the mean skin temperature in atopic dogs does not significantly differ from healthy dogs in most regions, the higher temperature observed in the tail area suggests a localized variation that may be relevant to the pathophysiology of atopic dermatitis.

Considering the lesion of dogs with caD, affected body sites are classically distributed at head, extremities, axillae, and ventral abdomen [49]. Within years, typical lesions and affected regions of caD have been evaluated for both diagnosis and disease various clinical scorings have been developed for following [17,50,51]. In our study, body site lesions and pruritus evaluation and scoring were performed with CADESI-4 [17] and PVAS [18]. These were used for assessing the relationship between clinical findings and skin barrier at a given site. Observed by Marsella et al.[31] CADESI-03 was found to be correlated with TEWL and PVAS (P<0.05) without hydration. Also, it was found that skin barrier evaluated parameters (capacitance, pH, TEWL, erythema and absorbance) had a low correlation with the CADESI-4 [7]. Contrary to expectations, our study found no correlation between biophysical parameters and clinical scores (CADESI and PVAS) in atopic dogs.

There are some limitations to this study, such as a limited number of atopic dogs, differences in age, breed and diet of dogs included in the study, as well as the inability to demonstrate a clear relationship between the selected biophysical parameters and the specific site of the lesion.

We generated a regional map of atopic dogs by non-invasively measuring a range of biophysical parameters. These fundamental measurements provide a foundation to build upon for offering a practical approach to the disease. Our findings suggest that these parameters could be utilized to identify skin barrier dysfunction in dogs affected by atopic dermatitis.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available to the corresponding author (H. Erdogan)

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Ethics Approval: Ethical approval was obtained from Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University (No: 64583101/2024/22). Additionally, comprehensive information was provided to the owners of all dogs included in this study, and written consent was obtained from them.

Competing Interest: Authors declare that have no relevant financial or non-financial interests to disclose.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: HE, SE and KU designed and conceived of the study, HE, SE and KU performed corneometer analysis and collected data, HE performed the statistical analysis, HE and SE reviewed the literature and wrote the article. KU wrote and supervised all part of the study. All authors revised and approved the final version of the manuscript.

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Research Article

The Influence of Body and Metabolic Parameters in the Period Before Sexual Maturation in Heifers on the Lameness Score During the First Lactation

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Abstract

The aim of this research was to examine the relation between body and metabolic parameters in 105 young Holstein-Friesian cows in the period before puberty and locomotion score (LS) in the first lactation. During the examination of heifers (6-12 months of age) body condition score (BCS), body measurements and growth rate were determined. The blood to determine metabolic and hematology profile. The LS was evaluated monthly during the first five months after calving. Multivariate analysis and principal component analysis stated that selected body conformation parameters, blood parameters and LS were interrelated. Simple linear trends and/or complex LS prediction models indicated a positive correlation between values of LS and BCS, body growth, bilirubin, aspartate-aminotransferase, glucose, calcium, nonesterified fatty acid, leukocytes, granulocytes and negative correlation with albumin and cholesterol ($P<0.01$). A positive correlation existed between LS in different months of the study ($P<0.001$). Prediction of LS was significantly better ($P<0.0001$) using complex models ($r=0.736$-0.905) that include body measures, blood parameters, and LS in the first month compared to models with individual parameters ($r=0.547$-0.757). Predetermination of cows that will develop lameness problems such as LS>2 or LS>2 using two consecutive measurements are possible based on metabolic parameters. LS and lameness occurrence in the first lactation could be predicted in heifers very early, even before reaching sexual maturity, using blood parameters and body measurements.

Keywords: *Blood parameters, Body parameters, Heifers, Lameness score*

Introduction

Lameness is one of the most common diseases on farms, and in the absence of the reproductive problems and mastitis, the most frequent problem was observed [1-3]. The incidence of lameness has increased since the 1980s, and the prevalence has been considered to range from 6 to 42%. In addition, every animal is periodically or chronically exposed to lameness biennially for various reasons [4,5].

Lameness occurs under the influence of numerous risk factors [6,7]. A more recent meta-analysis and systematic review by Oehm et al.[8] using 53 manuscripts from a database of 1941 manuscripts identified 128 factors that were associated with lameness, and the most significant ones were the body condition score, excessive hoof growth, number of days of lactation, herd size and parity. The period from calving to the onset of lameness is related to prophylactic measures, hoof conformation and the occurrence of infectious or non-infectious lesions [9]. The lactation number and the period of lactation are significant risk factors for lameness, so Kougioumtzis et al.[10] determined that locomotion and lameness problems, during the first lactation, were less pronounced just before and immediately after calving, and that they increased as lactation progressed. Ristevski et al.[11] and Ristevski et al.[12] demonstrated that cows with milk production above 30.9 kg/day showed a higher risk of chronic lameness. Also, a suboptimal body condition score (BCS <2.5 or >3) at the peak of lactation increases the probability of lameness. The same authors showed that metabolic factors such as an increase in BHB, LDH, or lower triglyceride values were also very significant for the occurrence of lameness, and exposure to a greater number of factors exhibited an additional increase in the risk of lameness

The aim of this study was to examine the relationship between the physical and metabolic characteristics of heifers aged 6-12 months and the onset of lameness in the first lactation. Additionally, the study aimed to determine whether the lameness score during the first lactation, in addition to the heifers' physical and metabolic traits, could be used to predict lameness in subsequent lactation months.

Material and Methods

Ethical Statement

The study protocol was approved by the Institutional Ethics Committee of the University of Novi Sad (protocol code IV/2017/02).

Animals and Management

One hundred and five Holstein heifers aged 6-12 months were included in the experiment. The heifers were reared in standard group housing conditions. All the heifers were from the same farm. They were under the constant supervision of a veterinarian. Water was available ad libitum, and feeding was carried out according to the standard for heifers, which ensures a gain of 700-750 g/day. The meal is based on meadow hay, corn silage, corn kernels and sunflower meal with the addition of a complete mixture. A typical meal for heifers aged 6-12 months included 1.5 kg of alfalfa hay, 5 to 10 kg of corn silage (from 6 to 12 months of age), 2.0 kg of sugar beet noodles, and 1.5-2.0 kg of concentrate (from 6 to 12 months of age). The meal contained the following composition: 6-7.5 kg of DM, NEL 40-48 MJ, SP 800-900 g, NDF 25%, NDF from roughage 19%, ADF 19%, and fat around 3%.

In this period, the heifers get used to a coarse meal, so the share of hay and silage increased in 12-month-old heifers compared to 6-month-old heifers.

Lameness Assessment

The Sprecher diagnostic scale of 1 to 5, where a score of 2 or higher indicates different levels of lameness, by descriptive and visual guide was considered for lameness evaluation *(https://open.lib.umn.edu/largeanimalsurgery/chapter/bovine-orthopedics/).*

Body Condition Score and Measurements

The measurement was performed by adspection of heifers and palpation of prominent body parts. For this purposes recommendations from Elanco Animal Health Bulletin Al 8478 (Rev.9/96) were used. Body mass was measured on a farm scale, and growth was determined on a monthly basis.

Laboratory Blood Analysis

Blood was taken by coccygeal puncture. The samples were taken in vacutainers with the addition of EDTA for hematological analysis and in plain tubes for serum separation for biochemical parameters. Hematological parameters included red blood cells (RBC), hemoglobin (HGB), mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). Metabolic parameters in blood associated with metabolic stress were: non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), glucose, total bilirubin (TBIL), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), cholesterol (CHOL) and triglycerides (TGC). An automatic Chemray analyzer (Rayto, China) and standard kits (Biosystem, Spain) were used for biochemical analyses. Hematological analyses were performed with an automatic hematological counter Mek-6550 (Nihon Kohden, Japan).

Statistical Analysis

The relationship between the examined parameters was determined in order to analyze the possibility of establishing certain hypotheses about influence of BCS, body parameters and blood metabolic parameters on LS. This was done using multivariate statistics and principal component analysis. When dependence between the examined parameters was determined, the standard correlation analysis between the examined parameters. For this purpose, Pearson's correlation coefficient was considered. Complex linear models at three levels were carried out: models that included only body measurements, models that included only blood parameters, and models that included body measurements + blood parameters + health status (lameness score in the first month after calving). A linear regression model was applied, and the outcomes are displayed as a partial correlation of each parameter independently, highlighting the significance of each parameter on the LS prediction model as a whole. The difference in model quality was determined by comparing the coefficients of the entire model and using the Fisher transformation method.

Problematic cows - those with average LS above 2 and those with a score above 2 in two consecutive measurements - were identified using a logistic model. Cows that had problems were marked with a value of 1, while cows without such problems had a value of 0. The measure of discriminatory ability was shown by the area under the ROC curve.

The software at the link *https://biit.cs.ut.ee/clustvis/* was used for multivariate analysis, while other statistical analyzes were performed in the statistical program SPSS (IBM, USA).

Results

Multivariate analysis and analysis of principal components was showed the association between BCS and blood

parameters with LS in the first five months of the first lactation. The heat map shows that LS values and the percentage of problematic cows, as well as BCS values and many blood parameters changed in a relatively regular same or opposite direction with LS intensity. Further analyses of the main components showed that the use of selected parameters were enabling the differentiation of cows with different locomotor scores, as well as that discrimination can occur on the basis of BCS, but not on the basis of the age of the heifers when body measurements or blood sampling was considered. The results are shown in *Fig. 1*.

The relationship between the value of the locomotor score per month as well as the average value of the score was established. Tests indicated that there was a statistically significant positive correlation between all LS values from the first to the fifth month, as well as a high correlation of individual LS with the average LSAv. Correlations were of medium to strong intensity, and their value ranged from 0.346-0.793 (P<0.001). BCS generated a positive correlation (P<0.001) with the locomotor score and all problematic categories of heifers, except that it was not related to the category of heifers that had at least once a value of LS=3. Correlation between blood parameters and LS was also established in heifers. ALB showed a negative correlation with LSM4, LSM5, LSMAv, and the proportion of problematic heifers (with average LS more than 2 or LS in two consecutive measurements higher than 2). GLOB showed a positive correlation with LSM3, LSM4, LSM5, LSMAv and with the occurrence of heifers that have lameness problems such as high lameness score at level 4 or 5 and in which two consecutive measurements are higher than 2. TBIL and AST showed a statistically significant positive correlation with almost all LS and with the occurrence of heifers that are had problems with lameness. The correlation coefficient ranged from 0.2 to 0.45, and statistical significance was determined at the P<0.01 level for most connections. GLU shows a positive correlation with all LS (except LSM1) and all categories of problem cows, a correlation level of around 0.25 was reached with a statistical significance of P<0.01 for most connections. Ca showed a positive relationship

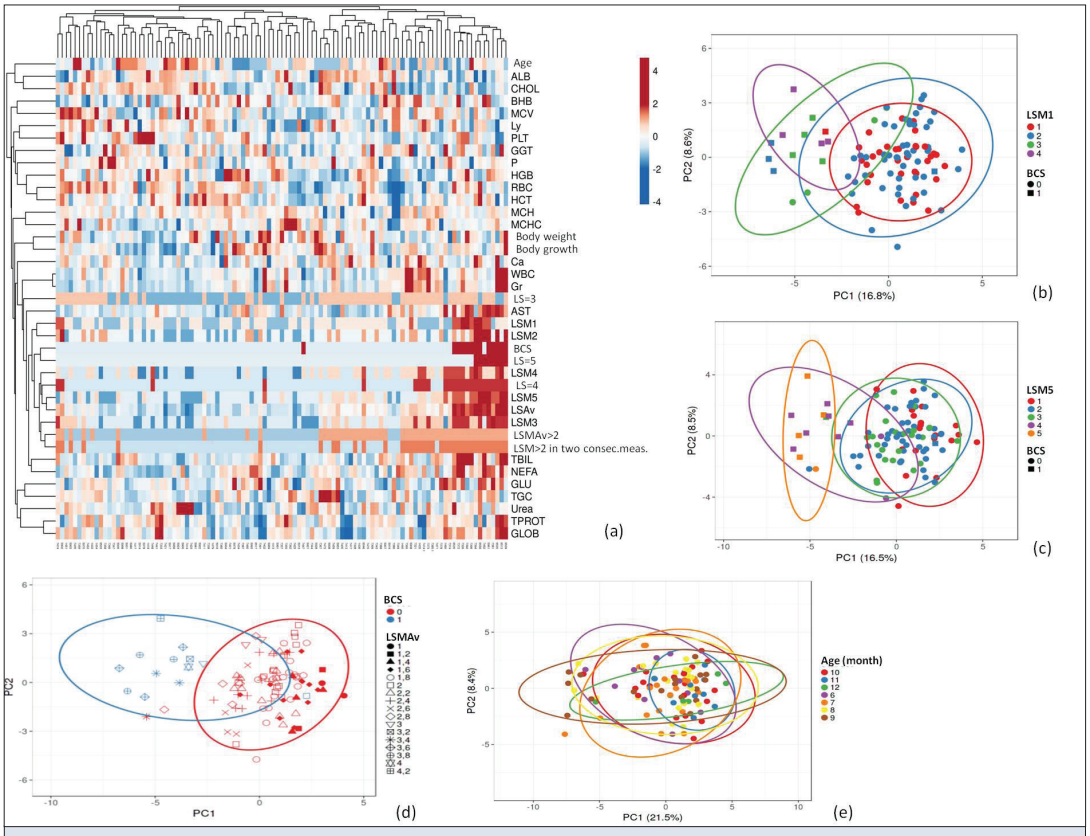

Fig 1. Heat map from multivariate analysis, intensity and direction of changes in locomotor score, body measurements and blood parameters in heifers (a). Principal component analysis and separation of heifers with different LS (b, c), BCS (d) and age (e) based on the values of the multivariate analysis of the investigated parameters. LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet

with LSAv as well as with certain categories of problem heifers (those with an average LSAv over 2 and which in two consecutive measurements had LS greater than 2), and these correlations were statistically significant from P<0.05 to P<0.01. CHOL was negatively correlated with LSM5, LSMAv and with heifers that had LS 4 or 5 at least once and that in two consecutive measurements had LS greater than 2), and these correlations were statistically significant from P<0.05 to P<0.01. Blood parameters such as NEFA, WBC and granulocyte (Gr) (which is reflected in the number of neutrophils) showed a positive correlation with all examined parameters of lameness (P<0.01). Hematological parameters of red bloodline showed no significant relationship with lameness in heifers. PLTs

Table 1. *Correlations between the examined parameters and their statistical significance*

Parameters	LSM1	LSM2	LSM3	LSM4	LSM5	LSMAv
LSM1						
LSM2	0.572***					
LSM3	0.346**	0.459***				
LSM4	0.372**	0.415***	0.528***			
LSM5	0.379**	0.432***	0.505***	0.570***		
LSMAv	0.679***	0.750***	0.761***	0.776***	0.793***	
BWG	0.160	0.150	0.163	0.061	0.210*	0.199*
BCS	0.539**	0.436**	0.563**	0.543**	0.629**	0.723**
TPROT	0.021	0.032	0.081	0.092	0.032	0.061
ALB	-0.171	-0.134	-0.192	-0.201*	-0.261**	-0.258**
GLOB	0.151	0.132	0.232*	0.255**	0.232*	0.271**
TBIL	0.427**	0.399**	0.385**	0.398**	0.413**	0.534**
AST	0.232*	0.224*	0.309**	0.261**	0.450**	0.401**
GGT	0.051	0.012	0.082	0.102	0.123	0.101
GLU	0.132	0.246*	0.183	0.265**	0.297**	0.302**
Ca	0.174	0.262**	0.154	0.162	0.191	0.247*
P	-0.14	0.023	-0.082	-0.013	-0.062	-0.074
Urea	0.052	-0.064	0.014	0.101	0.021	0.033
CHOL	-0.152	-0.173	-0.248*	-0.191	-0.237*	-0.265**
TGC	0.093	0.151	0.064	0.012	0.041	0.091
BHB	0.054	0.062	0.024	0.103	0.172	0.112
NEFA	0.343**	0.254**	0.386**	0.345**	0.436**	0.473**
WBC	0.032	0.238*	0.403**	0.260**	0.280**	0.332**
Ly	-0.053	-0.043	0.043	0.013	-0.071	-0.033
Gr	0.052	0.255**	0.392**	0.256**	0.308**	0.345**
RBC	0.153	0.152	0.132	0.103	-0.021	0.132
HGB	0.112	0.033	0.101	-0.042	0.072	0.074
HCT	0.154	0.032	0.172	0.094	0.063	0.134
MCV	-0.013	0.054	0.171	-0.135	-0.171	-0.032
MCH	0.012	0.062	0.09	0.003	0.013	0.054
MCHC	-0.052	0.064	0.122	-0.034	0.084	0.052
PLT	0.236*	0.001	-0.101	-0.052	-0.121	-0.031

LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet
** P<0.05; ** P<0.01; *** P<0.001*

showed a positive correlation with LSM1. The results are shown in *Table 1*.

Models based on body measurements include the use of parameters such as breast girth, body length, weight, gain and BCS in the prediction of LS in heifers in the first five months after calving (LSM1-5) and LSMAv. The use of these parameters enables a statistically significant prediction of the locomotor score in each month as well as the average LSMAv with a correlation level of r=0.519-0.757 (P<0.001). The BCS value had the largest partial correlation and a dominant role in the model. Additionally, LSM5 and LSMAv positively correlate with body weight gain. The results are shown in *Fig.2*.

The blood parameter-based model enables statistically significant predictions of both monthly LS and LSAv, with a correlation level of r=0.604-0.787 (P<0.01 to P<0.001). Depending on the month of LS determination, blood parameters such AST, GLU, Ca, TBIL, NEFA, BHB, WBC, and Gr were statistically significant in the model. The results are shown in *Fig.2*.

Models based on body measurements + blood parameters + locomotor score in the first month include the use of all measured parameters in the prediction of the locomotory score in heifers from the second to the fifth month after calving, as well as the average score. The use of these parameters enables a statistically significant prediction of the locomotor score in each month as well as the average for all five examined months with a correlation level of 0.736-0.905 and a statistical significance of P<0.001. BCS plays a dominant role in the model, and parameters such as TBIL, GLU, Ca, NEFA, WBC, MCV, MCH or HCT have statistical significance, which depends on the month of the test, while LSM1 significantly correlates with LSM2 and LSAv. The results are shown in *Fig.2*.

The model with the lowest correlation with LS was the one that just included body measures. Models with blood parameters and body measurements also showed a stronger association, and models with blood parameters, body measurements, and health (LSM1) showed the strongest correlation with LS. When body measurements, blood parameters, and LSM1 were included in complex

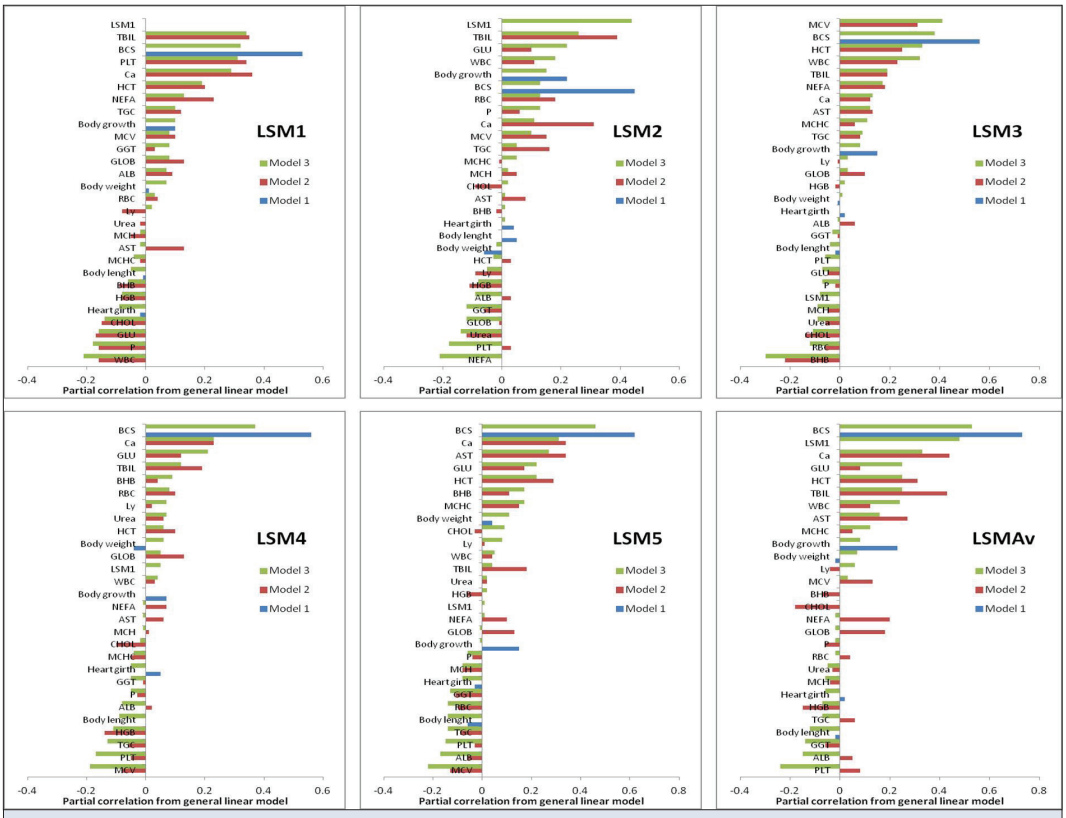

Fig 2. Partial correlations of parameters with LS obtained from GLM models including body measurements (Model 1), blood parameters (Model 2), body measurements + blood parameters + LSM1 (Model 3). (**Partial correlation** r>|0.15|, P<0.01). LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet

Table 2. *Efficacy of models by comparing their correlation coefficient between the predictor and the outcome variable via Fisher's r-to-z transformation*

Outcomes	Correlations in Model 1 (Body Measurements)	Correlations in Model 2 (Blood Parameters)	Correlations in Model 3 (Body Measurements + Blood Parameters + LSM1)	Significance of Difference		
				Model 1: Model 2	Model 1: Model 3	Model 2: Model 3
LSM1	0.547	0.681	0.736	NS	<0.001	NS
LSM2	0.519	0.633	0.758	NS	<0.001	NS
LSM3	0.600	0.741	0.794	NS	<0.001	NS
LSM4	0.593	0.604	0.706	NS	<0.001	NS
LSM5	0.647	0.699	0.793	NS	<0.001	NS
LSMAv	0.757	0.787	0.905	NS	<0.001	<0.001

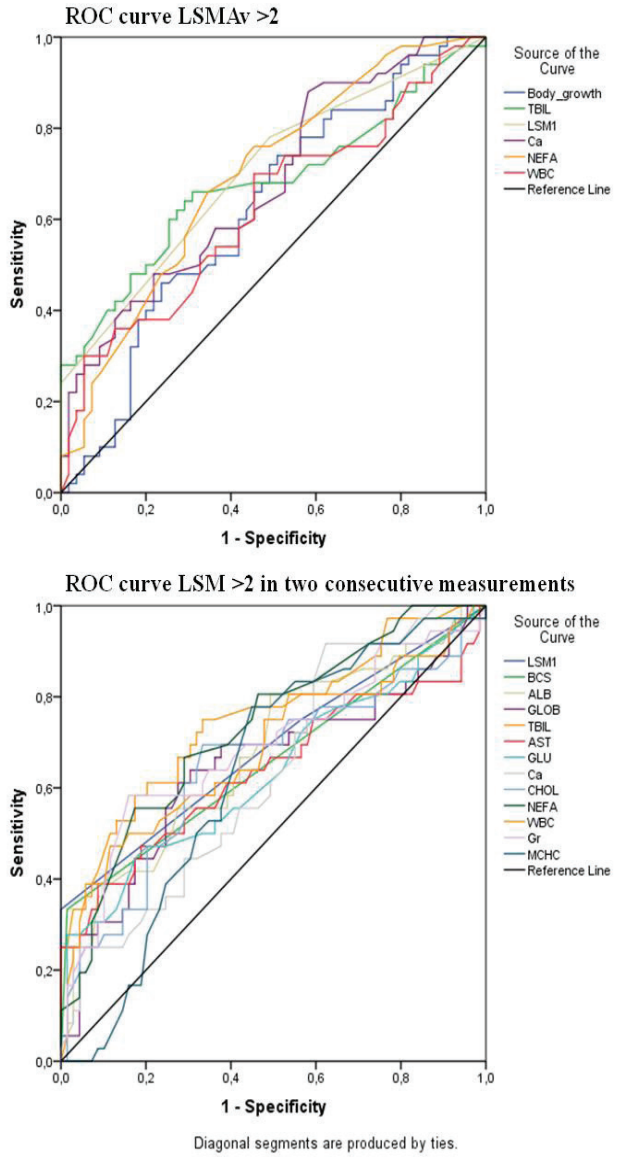

Fig 3. The ability of the tested parameters to discriminate cows that will not have problems with lameness and that will have the following problems: LSMAv >2 and LSM>2 in two consecutive measurements. LSM1: Locomotion score in month 1, BCS: Body condition score, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GLU: Glucose, Ca: Calcium, CHOL: Cholesterol, NEFA: Non-esterified fatty acids, WBC: White blood cells, Gr: Granulocyte, MCHC: Mean corpuscular hemoglobin concentration

models (r=0.736-0.905) versus individual parameter models (r=0.547-0.757), the prediction of LS was much greater (P<0.0001). The results are shown in *Table 2*.

Body and blood parameters assessed before puberty can aid in the early detection of heifers with substantial lameness issues, such as LSMAv >2 or LSM >2 in two consecutive monthly measurements. Heifers with an average LSMAv >2 can be identified and differentiated from those with lower average values using LSM1, body gain, TBIL, Ca, NEFA, WBC, and Gr. The ROC AUC value ranged from 0.62-0.7 (P<0.05 to P<0.01). The parameters LSM1, BCS, ALB, GLOB, TBIL, AST, GLU, Ca, CHOL, NEFA, WBC, Gr, and MCHC can be used to distinguish heifers with lameness problems from those without. ROC AUC values varied from 0.63-0.75 (P<0.05 to P<0.01). The results are shown in *Fig.3*.

Discussion

LS were examined during our experiment on a monthly basis for the first five months of the first lactation of heifers and a positive correlation was demonstrated. The percentage of different LS grades in our sample was: 19% for LS=1.48% for LS=2.25% for LS=3.9% for LS=4 and 5% for LS=5. Our results roughly match the results obtained by Matson et al.[5]. The author [11] used a similar research model to ours and found that the average lameness score (LS) was 2.9±1.1. LS increased in the first and second months of lactation, decreased in the third and fourth months, and increased in the fifth and sixth months.

In addition, a positive link was observed between the intensity of lameness from the first to the fifth month of lactation. Our findings agree with those obtained by the cited author. Based on research conducted on Danish dairy cows, there was a substantial relative risk that lameness from the previous lactation could occur in the subsequent one [13]. There has also been shown a correlation between several forms of hoof disease; for example, hoof ulcers and pre-existing interdigital dermatitis, and solar ulcers and interdigital dermatitis in the future [14-16]. Large-scale investigations done in France

on dairy cows have established the link between lameness and lactation [17]. Compared to non-lame cows, previously lame cows had a two- to three-fold higher risk for all types of lameness, according to a sizable database compiled by French researchers [18].

Complex models determined that cows that exhibited lameness in the week immediately before calving were often lame in weeks 2 and 8 after calving as well. Daros et al.[19] conducted a prospective longitudinal study involving hundreds of cows during the dry season. Randall et al.[20] presented their results from an eight-year study that included complete longitudinal data for over 1500 cows. The results indicated that 70-90% of lameness in cows was actually a repeated episode that had been previously present. Between 9 and 21% of lameness cases could be attributed to previous lameness that occurred >16 weeks before the risk period.

Metabolic factors were associated with the onset of lameness. In an interesting study [21], the use of a complete metabolic profile in predicting lameness and non-infectious hoof diseases was evaluated, and it was discovered that serum albumin and proteins significantly decreased prior to the occurrence of sole ulcers and sole bleeding, AST significantly increased in cows with bleeding and sole ulceration, and NEFA increased significantly prior to hemorrhage and/or sole ulceration. Additionally, lame cows had reduced levels of BCS, cholesterol, glucose, albumin, urea, Ca, and Mg [22].

Metabolic parameters such as NEFA, GLU, AST, or BCS were related to LS in our study as well, so our results match the ones obtained by the previously mentioned authors. The importance of metabolites such as NEFA and BHB was also demonstrated in primiparous cows that developed hoof lesions during mid-lactation, and blood was sampled retrospectively in the first weeks after calving [23]. The authors found that NEFA and BHB concentrations were significantly higher in the first weeks of lactation in cows that developed signs of lameness, and cows exhibited a more pronounced loss of body condition. Higher glycemia and an increased NEFA value indicated insulin resistance. During the period of positive energy balance, it is accompanied by a high insulin value, which all together reduces the production of keratin and disrupts the anatomy of the hooves, which increases the risk of lameness [24]. The greater the sensitivity to insulin and positive energy balance in the period before calving, the greater the negative energy balance, lipolysis and loss of fat depot after calving [25], which also reduces hoof fat pads and increases lameness. Our results demonstrate that higher body condition, higher glycemia and higher value of NEFA and BHB in the prepubertal period cause a higher lameness score in early lactation. Higher availability of sugar components even long before lactation can lead to the onset of lameness during lactation. It had been noticed that farms that used corn silage had more lameness cases than those not based on silage, and the oligofructose overload model proved to be extremely efficient for studying laminitis [26,27]. When heifers are being fed abundantly to achieve growth greater than 700 g/day, lameness occurs without clinically or morphologically visible causes [28]. All of the above is in accordance with our findings, which demonstrate that increased body growth and higher body condition in heifers before puberty mean higher LS after parturition. Blood calcium concentration is positively associated with LS. It is known that high levels of calcium in the blood weaken the bones due to the action of the parathyroid gland, and this effect is especially visible after calving and during lactation [29].

Models for evaluating the onset of lameness or evaluating the LS include the use of various indicators, such as body condition, metabolic parameters of milk and photographic parameters [30-32]. These models gave estimates that were most often at the level of 57-62%, and that percentage increased to a level of over 75% when there was a dichotomous classification of lameness or when a longitudinal assessment of lameness was performed. Our results coincided with the stated results of the researchers, so our model could be considered quite efficient in assessing the occurrence of lameness in heifers at the beginning of the first lactation, although we used parameters obtained from a time distant point in relation to the first calving, which was the period before puberty maturation and during intensive growth. Future research (to fully evaluate and upgrade the model) should include a larger number of farms with different zoohygiene conditions, different feeding methods, with heifers of different breeds, through different seasons, with heifers of different health status after calving, and so on, because all of these factors can have an impact on the model's quality and lameness predictions. Metabolic diseases should be studied in depth because of their relationship to body condition and metabolic parameters [33-35], as this allows us to precisely quantify the influence of body condition, disease, and metabolic variability on the onset of lameness.

The test results demonstrate that based on body condition and blood parameters in the period before puberty as well as the locomotion score in the first month of the first lactation, the value of LS in the first five months of lactation as well as the average value of LS can be predicted. Metabolic parameters and assessed body condition exhibit a significant correlation with LS values, and can indicate the ability to classify cows into those that will and will not have problems with lameness in the first lactation. These results confirm that in the early development of heifers, before sexual maturity, using

their body and metabolic characteristics, the value of LS can be predicted during the first five months of the first lactation.

Declarations

Availability of Data and Materials: Data will be available at https://www.open.uns.ac.rs/ after dissertation defense of first author (N. Zahirović).

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Research Article

Effects of Statins on Skeletal Muscle Contractile Properties in Rats

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Abstract

The aim of this study was to investigate the short-term effects of statins on rat skeletal muscle with the use of electrophysiological methods. A total of 28 adult male Wistar albino rats were given atorvastatin, rosuvastatin, and pravastatin (40 mg/kg each) intragastrically for four weeks. At the end of the study, the gastrocnemius muscle was isolated from the surrounding tissues under deep anaesthesia. It was tied with silk thread in the Achilles tendon area and then connected to an isometric force transducer. The contractile properties of the gastrocnemius muscle were assessed. Serum levels of total cholesterol and creatine kinase (CK) were measured. The muscle tissue samples were stained with hematoxylin eosin and histopathologically evaluated under light microscope. The findings revealed that cholesterol levels were significantly lower in all groups except the control group, whereas CK levels were higher in the statin groups than the control group. All three statins decreased the contractile strength and caused fatigue quickly in the gastrocnemius muscle. Histopathological changes in gastrocnemius muscle were more related to early moderate necrosis in rats that were received statins. This study demonstrates that all three statins decrease the ability of skeletal muscle to generate force and its resistance to fatigue in rats.

Keywords: Creatine kinase, Myopathy, Rat, Skeletal muscle contractility, Statin

Introduction

Cardiovascular diseases have remained one of the most important health problems for all over the world. The total number of cases increased from 271 million in 1990 to 523 million in 2019, while the number of related deaths increased from 12.1 million to 18.6 million [1]. Statins significantly reduce the incidence of coronary heart disease, being the most effective hypolipidemic compounds that reduce mortality in patients with cardiovascular diseases [2-4]. A study that was conducted in the US from 2003-2012 reported that approximately 71% of adults diagnosed with cardiovascular diseases, 63% of those diagnosed with diabetes and 54% of patients with hypercholesterolemia were taking cholesterol-lowering medications [5].

Statins are very well tolerated by most patients. However, 10-12% of patients may develop muscle-related side effects [6]. Other side effects of statins include new-onset type 2 diabetes, neurological and neurocognitive effects, hepatotoxicity, renal toxicity, and the adverse effects on gastrointestinal, urogenital, and reproductive systems [7,8]. In a survey study conducted in France, muscle symptoms such as muscle pain, tenderness or weakness were reported in 10% of patients taking statins for the treatment of hypercholesterolemia and one third of these patients had to discontinue the treatment. The most prescribed statins are low dose rosuvastatin and atorvastatin. Soreness was the most described symptom and many patients reported stiffness during exercise, cramps, weakness, or loss of strength. About 38% of patients reported that their symptoms prevented even moderate exertion during daily activities, while 42% of patients experienced significant interference with daily activities [9].

Lovastatin, pravastatin, and simvastatin are grouped as type 1 statins, while fluvastatin, cerivastatin, atorvastatin, and rosuvastatin are grouped as type 2 statins [10]. The side effects of statins may vary depending on the metabolic profile of statins. Moreover, other medications taken in combination may interfere with the mechanisms responsible for the metabolism or clearance of statins, leading to increased statin exposure and a significantly increased risk of myotoxicity [11].

This study aimed to investigate the possible short-term effects of three statins (atorvastatin, rosuvastatin, and pravastatin) from two different groups mentioned above,

widely used in clinical practice, on the skeletal muscles of rats with the use of electrophysiological methods.

Material and Methods

Ethical Statement

This study was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (Approval no: 64583101/2018/054).

Animals

A total of 28 male Wistar albino rats weighing ~350 g were used. The rats were housed in a research room with a 12/12 light/dark cycle, a temperature of 22±2°C, and 50-70% humidity. The rats were randomly divided into four groups (n=7/group) as atorvastatin, rosuvastatin, pravastatin, and control groups. Commercially available statins were purchased, grounded into powder, dissolved in saline, and administered via the intragastric gavage at a dosage of 40 mg/kg per day for four weeks. The control group received equal volume saline for the same period. The animals were weighed weekly throughout the experiment.

Muscle Preparation

At the end of the experiment, the rats were anesthetized with a combination of ketamine HCl (60 mg/kg) and xylazine HCl (10 mg/kg) and placed on a warm pad to help regulate their body temperature when they are under deep anaesthesia. The rats were then positioned on their right side and their tail and right hind leg were immobilized with flaster. The left hind leg was shaved, the skin was cut (between the lumbar vertebrae and the achilles tendon) and separated from the underlying tissues by using blunt dissection. The gastrocnemius muscle was isolated from the surrounding tissues and ligated with a silk thread at the achilles tendon area. The achilles tendon was then connected to an isometric force transducer (BSL, SS12LA, BIOPAC®, USA) [12]. After this, the stimulator (BSLTSMA, BIOPAC®, USA) was set to deliver one stimulus at a time (0.5 ms and 0.5 V). The stimulus was increased until the twitch amplitude remained constant. The maximum voltage was determined to be 3 V. The optimal length was determined by gradually stretching the muscle by 1 mm after each stimulus until the twitch amplitude was stabilized. This length was used for all subsequent measurements. The following parameters were determined from the recordings: single twitch force (g), time to peak twitch (ms), half relaxation time (ms), force-frequency relationship (muscle responses obtained by nerve stimulation at frequencies of 10, 20, 40, 60, 80, and 100 Hz for 0.5 ms and a total of 200 ms), maximum isometric tetanic contraction (the frequency with the strongest contraction was determined from 10, 20, 40, 60, 80 and 100 Hz frequencies), and muscle fatigue protocol (50 Hz frequency, 0.5 ms stimulation duration, and 300 ms). This procedure was repeated every second for five minutes, and the values for fatigue initial force (g) and fatigue duration (s) were calculated based on these data [13]. The muscle was allowed to rest for 5 min following the muscle fatigue protocol. The single-twitch protocol was performed again to determine the value of the amplitude of the recovery period (g).

Biochemical and Histopathological Analyses

After evaluating the contractility properties of the muscles, the animals were euthanized following intracardiac blood collection. Using commercial kits, the total cholesterol (Abbot® 7D62-21, USA) and creatine kinase (CK) levels (Abbot® 7D63, USA) were measured in the serum. The muscle tissue samples were stained with the hematoxylin and eosin and evaluated under a light microscope.

Statistical Analysis

The data were analyzed using IBM SPSS Statistics for Windows, version 19.0 (IBM Co., Armonk, NY, USA). Mean and standard error of the mean were presented. The distribution of the obtained data was evaluated using the Shapiro-Wilk test. Changes in body weight were analyzed by repeated measures ANOVA, while other variables were analyzed by one-way ANOVA followed by Duncan's post hoc test.

Results

As shown in *Fig. 1*, the body weights of all groups were normally distributed and there was no significant difference among groups at the beginning of the experiment (P=0.437, F=0.931). The differences among the groups remained statistically insignificant throughout the experiment while the body weights of the animals were seen to increase by time (P=0.026, F=4.141). Cholesterol levels were significantly lower in the rats that received statins than those of the controls. In addition, atorvastatin led to further reduction in cholesterol levels compared

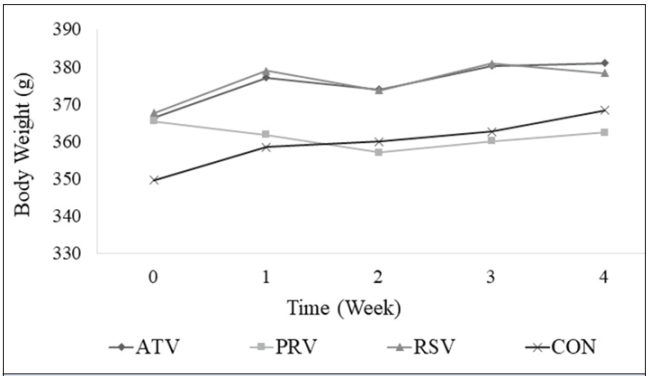

Fig 1. Body weight changes of the rats (g). The body weights of the animals were seen to increase by time (P=0.026).

Table 1. *Cholesterol and creatine kinase levels (mean ± SD)*

Parameters	CON	ATV	RSV	PRV
CHL (mg/dL)	116.06±3.61[a]	47.96±2.44[b]	75.23±4.81[c]	68.16±1.89[c]
CK (U/L)	626.50±6.02[a]	1489.25±163.66[c]	998.00±43.62[b]	707.75±54.31[ab]

Values in the same row *with* different *superscript* letters *differed significantly (P<0.001).*
CON: Control group; ATV: Atorvastatin group; RSV: Rosuvastatin group; PRV: Pravastatin group; CHL: Cholesterol; CK: Creatine kinase

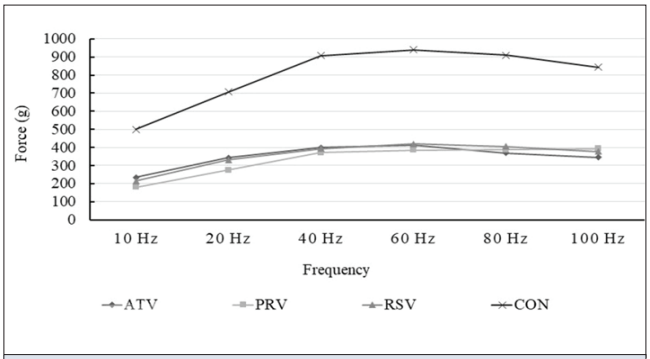

Fig 2. Values of the force (g)-frequency (Hz) of the gastrocnemius muscle. The control group differs significantly from the other groups at all measurement points (P<0.001).

Table 2. *Contractile properties of gastrocnemius muscle (mean ± SD)*

Parameters	CON	ATV	RSV	PRV	P Value
TPT (msec)	47.50±1.68[a]	36.57±3.41[b]	40.17±3.12[ab]	40.75±2.13[ab]	P<0.05
HRT (msec)	34.00±1.90	31.14±2.03	29.67±2.58	30.25±1.79	NS
STF (g)	535.96±50.99[a]	194.90±14.00[b]	181.17±22.63[b]	157.89±26.35[b]	P<0.0001
MITC (g)	998.67±77.79[a]	438.94±52.58[b]	437.21±42.95[b]	396.97±67.54[b]	P<0.0001
FD (s)	53.28±5.66[a]	16.21±1.12[b]	17.88±3.66[b]	14.48±3.25[b]	P<0.0001
FIF (g)	815.53±76.55[a]	309.78±60.86[b]	358.73±38.90[b]	354.31±65.80[b]	P<0.0001
ARP (g)	198.69±32.85[a]	127.64±13.72[ab]	81.24±24.63[b]	70.03±12.09[b]	P<0.01

Values in the same row with different superscript letters differed significantly. CON: Control group;; ATV: Atorvastatin group; RSV: Rosuvastatin group; PRV: Pravastatin group. TPT: Time to peak twitch; HRT: Half relaxation time; STF: Single twitch force; MITC: Maximum isometric tetanic contraction; FD: Fatigue duration; FIF: Fatigue initial force; ARP: Amplitude of the recovery period.

to rosuvastatin and pravastatin (P<0.001, F=36.634). CK levels in the rats, received statins (especially atorvastatin and rosuvastatin), were higher than the controls (P<0.001, F=19.210) *(Table 1)*.

Single twitch force of the rats that were received statins decreased compared to the controls (P<0.0001, F=30.128). The difference regarding time to peak twitch was significant between the control and atorvastatin groups (P<0.05, F=3.302), whereas the difference among groups in respect of half relaxation time was not significant (P=0.446, F=0.919). The statin administration caused a reduction (P<0.001) in force at all frequency ranges that were used in the force-frequency evaluation *(Fig. 2)*. The initial force and duration of fatigue were also reduced in the statin-received rats compared with the controls (P<0.0001, F=14.215, F=23.654 respectively). Single twitch force, which is the recovery time value taken after fatigue, was found to decrease in statin-received rats. The difference was significant in the rosuvastatin, and pravastatin groups compared to the control group (P<0.01, F=6.164), whereas the difference between the atorvastatin group and other groups was not significant *(Table 2)*.

The transverse and longitudinal sections of the muscle tissue, stained with the hematoxylin and eosin, were analyzed under the light microscope *(Fig. 3, Fig. 4)*. Necrosis was objectively scored as minimal (1)-up to %10 individual fibers involved in the whole area; mild (2)-up to 20% of fibers in the area; moderate (3)-up to 50% of fibers in the area; or severe (4)-greater than 50% of fibers in the area [14]. Degenerative necrotic changes in the muscles were mild in the rats, treated with atorvastatin and rosuvastatin, and moderate in the rats treated with

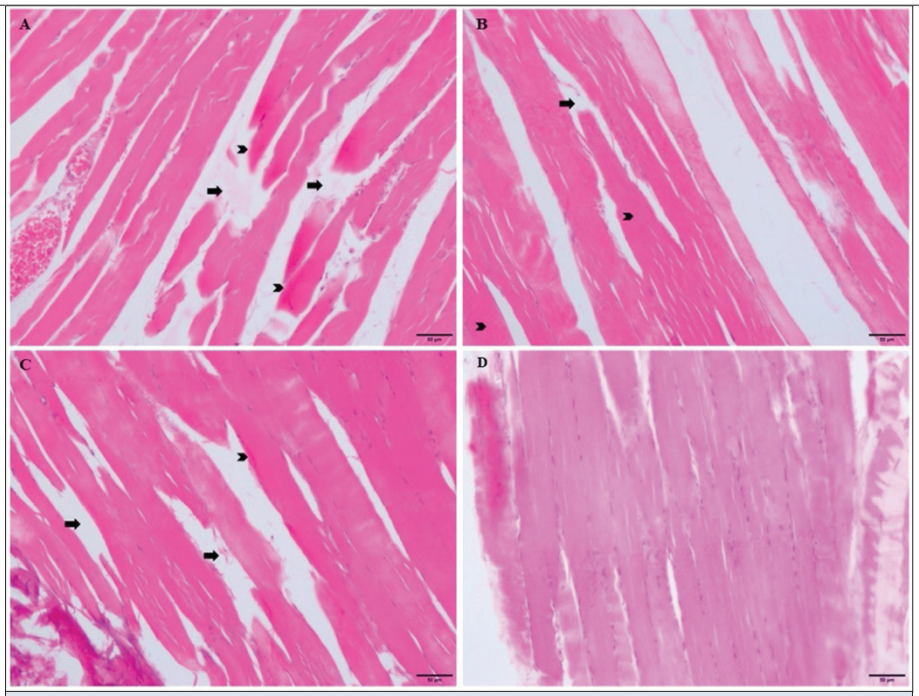

Fig 3. A-D: Cross-section of the gastrocnemius muscle. Atorvastatin, rosuvastatin, pravastatin, and control group, respectively. *Thick arrow:* myofibrillar rupture; *arrowhead:* hyper-eosinophilic fibres. H&E staining, scale bar: 50 µm

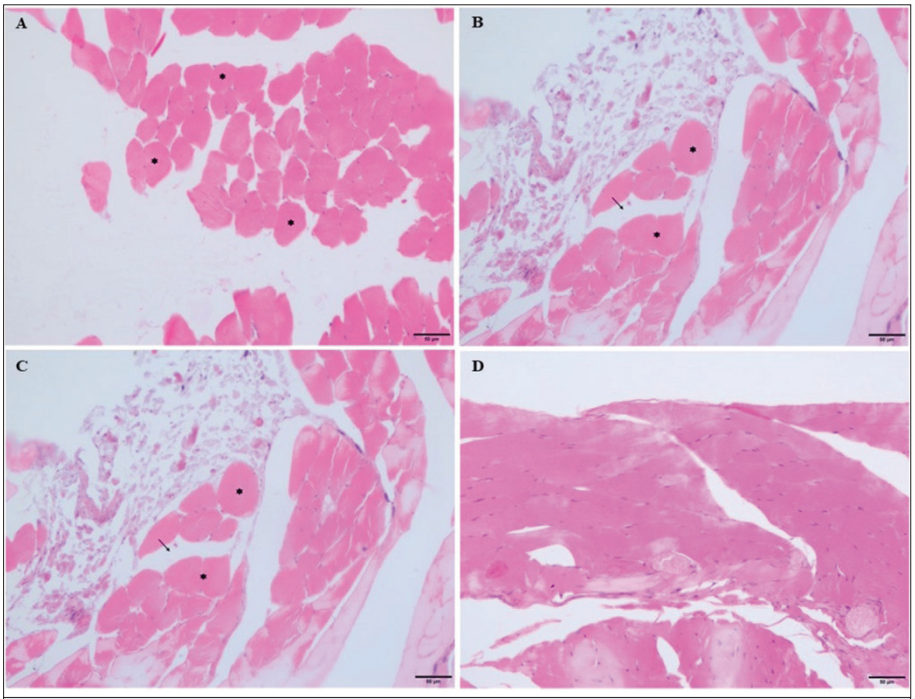

Fig 4. A-D: Longitudinal section of the gastrocnemius muscle. Atorvastatin, rosuvastatin, pravastatin, and control group, respectively. *: coagulation-like appearance, swelling; *thin arrow:* oedema between muscle bundles. H&E staining, scale bar: 50 µm

pravastatin. A marked disorganization of the muscle bundles, myofibrillar rupture, hypereosinophilic staining, coagulation-like appearance, fiber breakdown, and myofibrillar swelling and disintegration were observed in the longitudinal sections of the muscles in the statin-treated rats. In the rosuvastatin group, there was loss of transverse striation in some areas of the muscle. Additionally, there was dilation caused by edema between muscle bundles. Perivascular cell infiltration was seen in some muscle sections of the atorvastatin-received rats. Hypereosinophilic staining, coagulation-like appearance, fiber breakdown, and myofibrillar swelling were more

prominent in the pravastatin-received rats. Cross sectional evaluation revealed that the angular appearance of the muscle bundles disappeared, became rounded and separated. Hypereosinophilic staining and a coagulated appearance were observed in some of the transverse sections in the atorvastatin-received rats. In addition, degeneration in muscle bundles was prominent in the pravastatin-received rats.

Discussion

Long-term low dose of statin does not have a negative effect on body weight [15] which is consistent with this study. The increase in body weight by time in this study is due to the ongoing development of the rats. In rats, all three statins caused a decrease in cholesterol levels compared to the control group. It has been reported that different statins either do not lower cholesterol levels [16,17] or cause a weak reduction [15] in normo-cholesterolemic rats. However, the selected statins in this study led to significant reductions in cholesterol levels. The differences between studies may be due to the fact that the cholesterol-lowering effects of statins vary between species and depend on the dosage. Pecoraro et al.[18] stated that statins reduced total cholesterol in different species and the reduction was greater in animals that were fed with a high cholesterol diet. It was found that lipophilic statins (cerivastatin, simvastatin, fluvastatin, atorvastatin, lovastatin, and pitavastatin) had significantly greater cytotoxic effects in cell culture than hydrophilic statins (pravastatin and rosuvastatin) in a concentration-dependent manner [19]. Previous studies in rats have emphasized that a particularly high dose of statins is required to induce muscle necrosis [14,20]. Degenerative changes in the muscles have been reported to occur after a certain amount of time, such as 43 days, after administering simvastatin at a dose of 60 mg/kg/day in rats [20]. It has been suggested that myopathy may be initiated as early as the first day of statin administration and the necrosis may develop as early as 5 days after administration [21]. However, the data obtained in this study revealed that there was no evidence of statin-induced muscle severe necrosis. In our study, changes in gastrocnemius muscle were more related to early mild to moderate necrosis. We found a more pronounced degeneration in rats that received pravastatin. In contrast to the findings in our study, Bergman et al.[22] did not observe significant changes in the muscles of the mice that received pravastatin. El-Ganainy et al.[23] observed mild to severe necrosis in 60% of rats received atorvastatin (100 mg/kg/day), and mild necrosis in only 10% of rats, received rosuvastatin (80 mg/kg/day), after three weeks of statin administration. The changes in CK levels were one of the findings that reflects the severity of muscle degeneration. The known mechanism for the release of CK is the damage in the muscle tissue or changes in the permeability of the myocyte membrane. The rats, received atorvastatin, had the highest CK levels, followed by those received rosuvastatin when compared to the control rats. The CK levels of the control rats were above the normal levels. The reason for this may be that the blood samples were collected immediately after the in situ muscle protocol. This could be the same effect through which extended and intense training can harm muscle tissue and lead to a rise in serum CK levels which can be caused by both metabolic and mechanical factors [24]. It was reported that CK values doubled in rats, received 100 mg/kg/day of atorvastatin compared to the control rats [25]. CK levels increased in rats treated with high dose rosuvastatin. Interestingly, the increase in CK was started after the first 10 days of treatment and returned to normal levels by day 16 [14]. The CK values of the rats in the pravastatin group were similar to those in the control group, although slightly elevated. However, pravastatin group also displayed more prominence histopathologic findings of muscle degeneration. It is worth noting that some patients with statin-induced muscle symptoms had normal serum CK levels, despite the presence of marked weakness and histopathologic myopathy findings [26]. Reijneveld et al.[27] found that CK levels were similar to the control group after administration of pravastatin to young rats. However, elevated plasma CK levels can be used as a biomarker of tissue histopathology and severity of muscle toxicity [28]. According to the aforementioned studies, it can be stated that there is an increase in CK levels depending on the dose of statin.

Simvastatin [20], rosuvastatin [14,29], and atorvastatin [25,30] have been shown to cause muscle necrosis in rats. However, the doses used in the studies mentioned above are higher than those used in this study. In addition, the severity of the lesions varies depending on the statin dose administered. The muscles contain a significant proportion of type IIB fibers [14,20,25], and in these muscles, type IIB fibers become necrotic first [14]. Each of the three sections that make up the gastrocnemius has a different fibre composition. The red part has a high proportion of type I (slow twitch) fibers and low oxidative capacity fibers are absent, and therefore this muscle part has a high degree of fatigue resistance. The mixed part, adjacent to the red part and separated from it by a fibrous septum, contains a low proportion of type I fibers and a high proportion of IIB fibers. The largest white part contains only IIB fibers [31]. Therefore, there might be a correlation between the metabolic nature of muscle fibers and their susceptibility to statin-induced necrosis and these changes can be observed in fibers without concomitant changes in contractile elements, the endoplasmic reticulum or other subcellular components, suggesting that different parts of the muscle may be affected [20].

All three statins decreased the contractile strength of the gastrocnemius muscles and caused them to fatigue quickly. It was also determined that the time to twitch peak was shortened in the atorvastatin group compared with the control group. The administration of atorvastatin in rats results in a decrease in exercise capacity and impairment of the mitochondrial respiratory chain complexes in skeletal muscle. The correlation between exercise capacity and muscular oxidative capacity suggests that atorvastatin-induced mitochondrial dysfunction is responsible for the reduction in exercise capacity [32]. It is expected that some aspect of the intracellular Ca^{2+} transient, such as the peak amplitude and/or duration of the Ca^{2+} transient, will correlate with the contractile response during an isometric twitch [33]. Statins have been found to affect calcium channels that are crucial for muscle contraction and other fundamental functions [34]. The disruption of calcium homeostasis by statins is thought to be due to impairment of mitochondrial respiration or disturbance of intracellular calcium signaling [35,36]. We suggest that the decrease in the time to peak caused by the administration of atorvastatin may be due to a disturbance in the homeostasis of calcium.

Within the statin groups, the amplitude of the recovery period was closest to the control in atorvastatin group. However, amplitudes were significantly decreased in other statin groups compared to the control group. Sustained physical exercise reduces the ability to produce voluntary force. After this period, a rapid recovery of strength occurs due to the restoration of central fatigue (usually within 2 min) and aspects of peripheral fatigue related to excitation-contraction coupling and muscle reperfusion (usually within 3 to 5 min). Muscle function may not fully recover for several hours due to prolonged impairment in intracellular Ca^{2+} release or sensitivity [37,38]. The muscle phosphocreatinine metabolic recovery rate is an index of muscle oxidative capacity in vivo. A longer recovery time indicates impaired oxidative phosphorylation and/or mitochondrial ATP synthesis. The exposure to statins leads to a significant increase in metabolic recovery time after exertion. This suggests that statins may impair mitochondrial oxidative function [32,39,40]. The reduction in exercise endurance in mice by atorvastatin has been linked to muscle mitochondrial dysfunction as a result of ubiquinone deficiency. Pravastatin does not have a similar effect both on ubiquinone levels and exercise endurance in mice. The difference in membrane permeability of statins is thought to be the reason for their effect on muscle ubiquinone levels [41].

In conclusion, this study demonstrates that all three statins decrease the ability of skeletal muscle to generate force and its resistance to fatigue in rats. While the severity of degeneration and impairments in the contractile properties of the muscle are similar, there are differences in recovery period and time to peak twitch, suggesting that there may be other potential mechanisms besides common ones. This study attempted to examine the effects of statins by mechanical assessment of the muscle. For further studies, it will be important to conduct comprehensive analyses and approaches to explain the underlying mechanisms and to consider the time factor.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author (C. Ünsal) upon reasonable request.

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Ethical Statement

This study was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (Approval no: 64583101/2018/054).

Conflict of Interest: The authors state no conflict of interest.

Author Contributions: CÜ designed this study, collected the data set and revised the manuscript; EKY, ANA, HÜ collected the data set and made contributions to results and discussion.

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Research Article

Geometric Morphometric Analysis of Scapula at Cats and Dogs

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Abstract

The scapula in quadrupedal mammals is a flat bone that connects the thoracic limb with the trunk above the shoulder joint. For this purpose, scapulae of 34 dogs and 23 cats were modeled using computer tomography. 9 Landmarks and 50 semilandmarks were used. The cat and dog samples differ in scapula shape, as do male and female cats. Centroid size has no apparent covariance with shape, and we did not find any difference between the two sexes in dogs. The scapula of cats was wider. The scapula of dogs was narrower and longer. Margo cranialis was more oval in cats. Angulus cranialis border was not clear in cats. Angulus caudalis was sharper in dogs. Spina scapulae was closer to caudal in cats. In cats, the fossa supraspinata was wider than the fossa infraspinata. Also, the collum scapulae was narrower in cats. The scapula of male cats was wider than that of female cats. In shape, the fossa supraspinata was wider in male cats. In male cats, the spina scapulae were more caudal. Angulus caudalis was wider in female cats. The most significant gender differences in dogs were in tuberculum supraglenoidale and margo caudalis. Male dogs had larger tuberculum supraglenoidale in shape. Margo caudalis was more caudal in male dogs. Geometric morphometrics was found to be effective in distinguishing the scapula of cats and dogs. In addition, this method can be useful in sex estimation.

Keywords: Cat, Dog, Geometric morphometrics, Principal component analysis, Scapula, Sex differentiation, Taxonomy

Introduction

The scapula is a plane, triangular bone that connects the thoracic limb to the trunk. This bone is surrounded by the powerful muscles of the thoracic limb. Facies lateralis of scapula contains spina scapulae. The spina scapulae divide the facies lateralis into two faces, fossa infraspinata and fossa supraspinata. Unlike other species, spina scapulae in dogs is located in the middle of the facies lateralis. The spina scapulae terminate with the acromion near the angulus ventralis. However, spina scapulae terminate as processus hamatus in dogs, and additionally as processus suprahamatus in cats [1,2].

The margins of the scapula are called margo dorsalis, margo caudalis, and margo cranialis. Margo cranialis is flat in animals such as cattle and horses, but oval in cats and dogs. Margo caudalis is thicker than the other margins, because the musculus triceps brachii comes out of the margo caudalis and creates some bumps on the bone. It has been said that about these three margins of the scapula one can distinguish between species. However, the distinguishing information for the margins of the scapula for cats and dogs was not found in the reference information [1-3].

Geometric morphometry, unlike linear morphometry, detects shape differences between groups and reveals shape differences with statistical methods [4-7]. The term 'shape' here can be defined as the appearance obtained when we subtract the size of the example [8]. It was stated in the reference information that the shape showed

greater variation among groups than in size [9]. With these features, geometric morphometry has been studied especially on bones in recent years, and the differences in structures between species and genders have been tried to be revealed.

Shape analysis in geometric morphometry is done by placing 'landmarks' on certain anatomical points. These points are homologous points applied in the same way to all samples used in the study [10]. These landmarks are classified into three types [11]. Type I are points that are clearly located, such as the junction of two structures. Type II can refer to the most extreme or most recessed parts of a structure. Type III landmarks, which can also be called semilandmarks, can be defined as landmarks determined on the basis of other landmarks and used along a curve or a boundary. These points are located on the x and y coordinates in the coordinate system in 2D analysis.

For taxonomy, osteological methods can be applied easily and the type and animal sex determination on the basis of bone can be easily done with reference supports [12-14]. These methods are important for the identification of bones examples as well as for archaeological bones [15,16]. The most commonly used element of skeleton in terms of taxonomy is the cranium [17-19]. There is a study showing that species and sex distinctions can be made for many skull-related specimens. However, in recent years, gender and species analysis has also been included in reference studies in bones except the skull [20-22]. Processus hamatus and processus suprahamatus are decisive for the distinction between cats and dogs. Moreover, the shape difference of the anatomical borders of the scapula will also provide important information for veterinary anatomy. In addition, some parts of the bone may be missing in areas such as archeology, causing difficulties in terms of taxonomy. In this respect, evaluating more than one point for bone separation can contribute to the reference information.

This study aimed to investigate the usability of geometric morphometric analysis in gender analysis.

Material and Methods

Ethical Statement

Before starting the study, an application was made to the Animal Experiments Local Ethics Committee Presidency of Istanbul University-Cerrahpasa Rectorate for the necessary permissions. Conditional approval received (Document Number: E-74555795-050.04-882352). Informed Consent Form was taken from the patient owners.

Animals

In this study, computed tomography images of the thoracic region of 34 cats and 23 dogs were used. The examined and imaged animals had no symptoms of any orthopedic disease. The cats and dogs used in the study, their average age and average weight are given in *Table 1* and *Table 2*.

Table 1. *The average age and weight of the cats used in the study*

Breed	Female	Male	Age (Year)	Body Weight (kg)
Mix breed	17	4	5.83	4.68
Ankara Cat	1	0	13	6
Van Cat	1	0	1	3.3
Scottish fold	0	3	1.17	3.53
Blue Point Siamese	1	0	1	3.6
British Shorthair	3	1	4.63	3.6
Persian	1	2	13	3.93

Table 2. *The average age and weight of the dogs used in the study*

Breed	Female	Male	Age (Year)	Body Weight (kg)
Mix breed	3	2	6	18.63
Beagle	1	0	13	15
English Bulldog	0	3	3.67	23.5
Chihuahua	1		10	5
Cocker Spaniel	1	1	13	16
German Shepherd	0	2	7	40
Siberian Husky	1	0	8.50	23
Labrador Retriever	0	1	9	36
Pekingese	0	1	11	6
Pomeranian	0	1	4.00	3.7
Pug	0	2	10.75	9.6
Rottweiler	1	1	10	33.5
Staffordshire Bull Terrier	0	1	1	20

Modeling and Acquisition of Images

Computed tomography scans were taken using Siemens (Somatom Scope vc30b) at the Animal Hospital, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa. Scanning parameters for all samples were 0.6 mm slice thickness, 110 kV, and 28 mA, total scanning time approximately - 14 sec. After the scanning process was completed, the images were transferred to the computer and the segmentation process was performed. 3D models of scapulae were made using the 3D Slicer (5.1.0 version) program. Soft tissues were removed from the image using the software.

Images were obtained from all samples from the same position and saved to the computer in ''pnp" format. 57

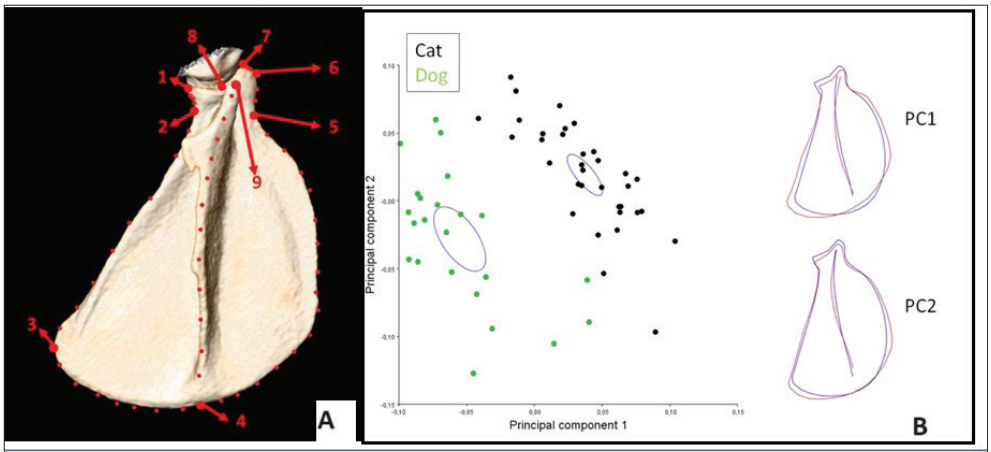

Fig 1. A: Landmarks (9) and Semilandmarks (50). *Red arrows:* landmarks, *small red points:* semilandmarks. **B:** Scatter plot of PC1 (30.1%) and PC2 (21.42%) of scapulae in lateral view for interspecies (left) (Confidence ellipses for means). Wire-frame warp plots of changes in the scapulas, as mapped by 9 land-marks, 50 semilandmarks (right). Blue outlines represent the mean shape configuration, while the red out-lines show the shape changes associated with the positive extremes of the PC axes

Table 3. List of landmarks applied to the scapula in lateral view

Landmark	Region
1	*Tuberculum infraglenoidale* 3 Semilandmarks
2	Caudal border of *collum scapulae* 8 Semilandmarks *(margo caudalis)*
3	*Angulus caudalis* 7 Semilandmarks
4	Middle point of *margo dorsalis* 15 Semilandmarks
5	*Incisura scapulae* 2 Semilandmarks
6	*Tuberculum supraglenoidale* 2 Semilandmarks
7	The most cranial point of *cavitas glenoidalis* 13 Semilandmarks
8	The middle point of the *cavitas glenoidalis*
9	The most ventral point of *spina scapulae*

images were converted to "tps" format using tpsUtil (version 1.74) [23]. 9 Landmarks (Type I) and 50 Semilandmarks (Type III) were used in the study *(Fig. 1-a)*. TpsDig2 (version 2.32) was used to insert the landmarks into the images [24]. The first, Landmarks were placed on all images *(Table 3)*. Then, 50 semi-landmarks were added. Finally, 'append tps curve to landmarks' was made using tpsUtil (version 1.74) again and semilandmarks were converted to Type I landmarks. Nomina Anatomica Veterinaria was used as a base for the anatomical terms of the landmarks used in the study [25].

Geometric Morphometrics

MorphoJ software was used for geometric morphometric analysis. Grouping operations were performed on the scapulae between species and sexes [26]. First, the differences in the shape of the scapula between cats and dogs were examined, followed by differences between sexes within species. Generalized Procrustes Analysis was performed before each group analysis. Then Principal Component Analysis (PCA) was applied. The shape and centroid size of these species and sexes were compared using Procrustes ANOVA. Discriminant function was used to reveal differences between cat and dog scapulae and between sexes.

Results

A total of 56 PCs were obtained to explain the morphological differentiation among cat and dog scapulae. PC1, which accounts for the most shape variation between species, explained 30.1% of the total variation *(Fig. 1-b)*. PC2 explained 21.42% of the total variation, while PC3 explained 12.64% of the total variation. The scatter plot of PC1 and PC2 of scapulae in lateral view is given in *Fig. 1-a*. Cats had higher PC1 values than dogs. The increase in PC1 value is correlated with a longer margo caudalis of the scapula. Also, when the PC1 value increased, spina scapulae were located more caudally. When the PC2 value increased, it was correlated with a longer caudal border of the scapula. PC1 would separate almost all cats and dogs. However, 3 dogs had a higher value for PC1 than the dog groups (Pekingese, male; mix, female and pug, male). One cat sample had a lower PC1 than the average cats (Mix, female).

Procrustes ANOVA results are given in *Table 4*. The cat and dog samples differ in scapula shape, as do male and female cats. Centroid size has no apparent covariance with shape, and we did not find any difference between the two sexes in dogs.

Table 4. *Centroid size and the shape of scapula standard deviations of quails*

Individuals	Measurement	F	P-Value
Species	Centroid size	0.88	0.3522
	Shape	17.62	<.0001
Sex (cat)	Centroid size	0.48	0.4942
	Shape	2.43	<.0001
Sex (dog)	Centroid size	0.25	0.6256
	Shape	0.51	1

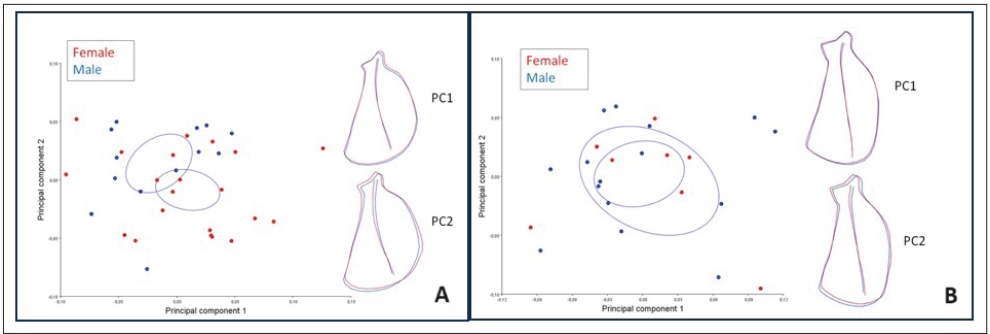

Fig 2. A: Scatter plot of PC1 (33.37%) and PC2 (16.77%) of scapulae in lateral view between sexes in cats (left) (Confidence ellipses for means). Wire-frame warp plots of changes in the scapulae, as mapped by 9 landmarks, 50 semilandmarks (right). Blue outlines represent the mean shape configuration, while the red outlines show the shape changes associated with the positive extremes of the PC axes. **B:** Scatter plot of PC1 (34.73%) and PC2 (19.23%) of scapulae in lateral view between sexes in dogs (left). (Confidence ellipses for means). Wire-frame warp plots of changes in the scapulae, as mapped by 9 landmarks, 50 semilandmarks (right). Blue outlines represent the mean shape configuration, while the red outlines show the shape changes associated with the positive extremes of the PC axes

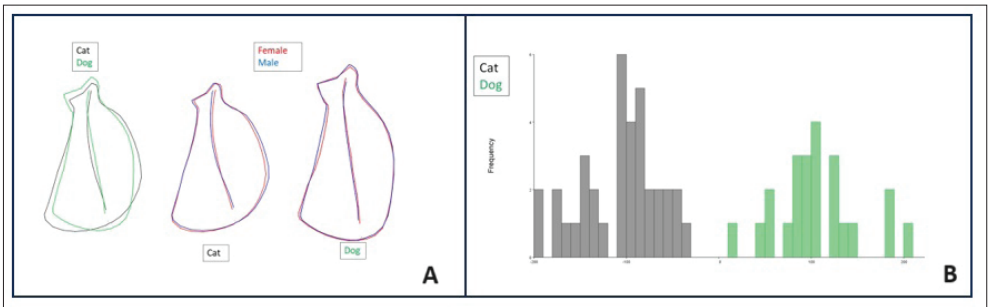

Fig 3. A: Wire-frame warp plots of changes in the scapulae. **B:** Distinctive scores and frequency for cats and dogs

A total of 32 PCs were obtained to explain the morphological differentiation between sexes in cats. PC1, which accounts for the most shape variation between sexes in cats, explained 33.37% of the total variation. PC2 explained 16.77% of the total variation, while PC3 explained 9.45% of the total variation. The scatter plot of PC1 and PC2 of scapulae is given in *Fig. 2-a*. The increase of PC1 is correlated with a wider tuberculum supraglenoidale. Females occupy the entire spectrum of PC1. Their values are not greater than those of males. However, males occupy mostly positive PC2 space, indicating a dorsoventrally elongated margo cranialis and a more posteriorly located spina scapulae in positive PC2 space.

A total of 21 PCs were obtained to explain the morphological differentiation between sexes in dogs. PC1, which accounts for the most shape variation between sexes in dogs, explained 34.73% of the total variation. PC2 explained 19.23% of the total variation, while PC3 had about 12.14% of the total variation. The scatter plot of PC1 and PC2 of scapulae in lateral view is shown in *Fig. 2-b*. No particular distribution was observed between males and females in the shape variation of the scapula.

The discriminant analysis result is given in shape difference *Fig. 3-a*. In shape, the scapula of cats was wider. The scapula of dogs was narrower and longer. Margo cranialis was more oval in cats. Angulus cranialis border was not

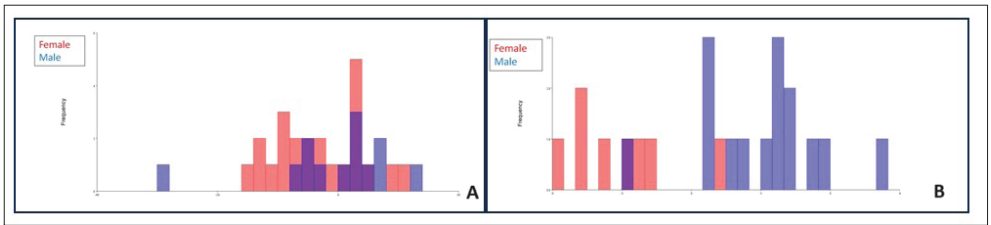

Fig 4. A: Distinctive scores and frequency for sexes for cats. **B:** Distinctive scores and frequency for sexes for dogs

clear in cats. Angulus caudalis was sharper in dogs. Spina scapulae was closer to caudal in cats. In cats, the fossa supraspinata was wider than the fossa infraspinata. Also, the collum scapulae was narrower in cats. According to the distinctive scores and frequency, it was seen that the cat and dog samples were completely separated *(Fig. 3-b)*.

The scapula of male cats was wider than that of female cats *(Fig. 3-a)*. In shape, the fossa supraspinata was wider in males. In males, the spina scapulae were more caudal. Angulus caudalis was wider in females. Distinctive scores and frequency for female and male cats are given in *Fig. 4-a*. There was no significant distribution in terms of gender of the samples used in the study.

The most significant gender differences in dogs were in tuberculum supraglenoidale and margo caudalis *(Fig. 3-a)*. Male dogs had larger tuberculum supraglenoidale in shape. Margo caudalis was more caudal in male dogs. Distinctive scores and frequency for female and male dogs are given in *Fig. 4-b*. There was no significant distribution in terms of gender of the samples used in the study like cats.

Discussion

The shape of the scapula in carnivores is strongly influenced by phylogeny, body size, and locomotion habits. [27]. Thus, when addressing the differences between cats and dogs, the type of locomotion in running dogs determines the long and slender scapula compared to the short and wide one in cats, adapted to climbing. The results of the study also supported this information. In shape, the scapula of cats was wider. The scapula of dogs was narrower and longer. The caudal border of the scapula in dogs is more developed than in cats, because of the active role of the m. serratus ventralis thoracis while running. The acromial part of the deltoid muscle is inserted on the processus hamatus. The morphometric analysis shows a more prominent processus hamatus in dogs than in cats. Given that the muscle acts in forelimb abduction, it may be assumed that this muscle is more active in dogs. Whilst sex distinction was statistically significant as a result of an analysis of the shape of the scapula in cats, no such differences were found in the dogs. This points to an aspect related to the greater allometric variability of shape in cats than in dogs [28].

In large ruminants, males have a wider basis of the scapula and higher spina scapulae, but the longitudinal dimension of the glenoid cavity is greater in females [29]. It is also reported [30], that basis scapulae are wider in male wild cats than in female individuals. It was also performed [31] a comparison between sexes in Van cats by using linear measurements of the scapula on the basis of three-dimensional reconstruction of computed tomography scans. The authors stated that the length of the spina scapulae, its height, the width of the fossa supraspinata, the width of the fossa infraspinata, the length of the tuberculum supraglenoidale, and the diameter of the cavitas glenoidalis were higher in male cats than in females. In this study, in which cats and dogs were used, geometric morphometry was used instead of linear measurements as in previous studies. By ignoring the size differences of the scapula, shape differences between species and genders were revealed. In the results of the shape analysis, it was seen that the scapula of male individuals for cats and dogs were wider than females.

In Van cats, both fossa supraspinata width and fossa infraspinata width were higher in males [31]. However, in the geometric analysis results, it was seen that the main difference here was in the fossa supraspinata. Because while margo caudalis was more caudal in shape, spina scapulae were in the same amount caudal in shape. While this ratio kept the width of the fossa infraspinale, wider fossa supraspinale was seen in males thanks to the margo cranialis, which is more cranial.

In a study it was observed [32], that processus hamatus exceeded the rim of the cavitas gelonidalis in wolves and foxes, but not in dogs. In Van cats, it was determined that processus hamatus slightly exceeded the border of cavitas glenoidalis as in wolves and foxes. In this study, we did not find that the processus hamatus extends beyond the outline of cavitas glenoidalis in dogs or cats.

In conclusion, although the cat and dog scapula were similar in shape, the difference between the two species could be revealed statistically with the geometric morphometrics. While there are not many size differences between scapulae of different breeds of cats, there are scapulae of different sizes among various breeds of dogs. The reason for this is that individual breeds vary considerably in body size. Regardless of this size variation,

the shape of the scapula in dogs remains similar. While traditional morphometric methods cannot establish a standard of linear dimensions for this bone, a standard shape can be determined even for dogs via shape analysis. This method seems to be more effective in cats than in dogs that this was also thought due to the low number of dog samples or the fact that there were too many various breeds, belonging to different morphotypes.

Declarations

Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author (D. Aydın Kaya) on reasonable request.

Funding Support: None.

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

Ethical Statement: This study was approved by the Animal Experiments Local Ethics Committee of Istanbul University-Cerrahpasa (Approval Number: E-74555795-050.04-882352).

Author Contributions: Conceptualization, Z.S.A. and Z.N.A.; methodology, O.S. and N.M.; software, D.A.K and E.O.; validation, E.O., T.S. and M.C.S.; formal analysis, O.S and E.O.; resources, Z.N.A. and Z.S.A.; data curation, Z.N.A. and Z.A.; writing-original draft preparation, M.C.S. and T.S.; writing-review and editing, N.M., M.C.S. and T.S.; supervision, Z.N.A.; project administration, D.A.K; funding acquisition, Z.N.A. All authors have read and agreed to the published version of the manuscript.

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Research Article

Effects of Zinc Oxide Nanoparticles on Pyruvate Dehydrogenase and Lactate Dehydrogenase Expressions and Apoptotic Index in Breast Cancer Cells

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Abstract

Zinc oxide nanoparticles (ZnO-NPs) are metal oxide NPs that have high cytotoxicity on cancer cells and low cytotoxicity on healthy cells. Breast cancer is the most frequent type of cancer-causing death among women worldwide. In this study, anti-cancer effects of ZnO-NPs were investigated. For this purpose, we treated the MCF7 and MDA-MB-231 breast cancer cell lines and human umbilical vein endothelial cells HUVEC cell line with 10 µg/mL and 20 µg/mL ZnO-NP. Anti-cancer effects of ZnO-NPs were evaluated with cell viability, apoptotic index and colony formation assays, and anti-Warburg effect were investigated by evaluating of pyruvate dehydrogenase (PDH) and Lactate Dehydrogenase A (LDHA) protein expressions. Results indicated that, ZnO-NP application did not have a cytotoxic effect on HUVEC cells, it had cytotoxicity on both breast cancer cell lines. However, MCF7 cells were more sensitive to ZnO-NP treatment. Administration of 20 µg/mL ZnO-NP reduced the survival of MCF7 cells by 62% and increased the apoptotic index by approximately 6 times. Additionally, ZnO-NP treatment inhibited the doubling times of cells and suppressed the colony-forming abilities of both breast cancer cell lines. Also, it was seen that ZnO-NP treatment increased PDH expression in MCF7 cells, where the apoptotic index was more induced. As a result, we have shown for the first time that ZnO-NPs affect the energy metabolism of cells by increasing PDH expression in MCF7 cells, thus increasing the apoptotic index. Our study, which observed the anticancer effects of ZnO-NPs on breast cancer cells, will also shed light on future experimental studies.

Keywords: Breast cancer, Lactate dehydrogenase A, MDA-MB-231, MCF7, Pyruvate dehydrogenase, Zinc oxide nanoparticles

Introduction

Cancer, the second leading cause of death worldwide, is a group of oncological diseases characterized by the irregular growth and proliferation of a single cell by the accumulation of different mutations as a result of impaired cell control. Breast cancer, on the other hand, is a type of cancer with a high prevalence among women [1]. There are different subtypes of breast cancer and the pathological classification of these subtypes is evaluated according to the expression of estrogen (ER), progesterone (PR), and human epidermal 2 (HER2) proteins by tumor tissue [2]. Breast cancers that do not have ER, PR, and HER2 protein expressions are referred to as triple negative cancer (TNBC), thus they are known as a more aggressive type for prognosis and curation. On the other hand, breast cancer that expresses hormone receptors is classified as a hormone receptor-positive (HR+) subtype and it has a better prognosis thus it is admitted as a subtype that easily takes under control [3]. Conventional treatment methods against breast cancer include radiotherapy, hormonotherapy, chemotherapy, and surgical interventions [4]. However, since tumor tissue can show heterogeneous mutation distribution, the bioavailability of conventional treatments is less than expected [5]. In this respect, the use of nanoparticles (NP) designed for treatment or diagnosis in the field of nanotechnology is a current area of research [6]. Various NPs based on organic, inorganic, lipid, protein, glycan, or

synthetic polymers are used to develop new therapeutic agents for use in cancer treatment. On the other hand, the use of commercially produced NPs in cancer treatments contributes to low toxicity by increasing drug efficacy [7]. In addition, numerous reports show that NPs have a high affinity for reaching specific organelles such as mitochondria and nuclei [8]. It is also known that rationally engineered NPs by modulating particle size, morphology, and surface modifications can also infiltrate hypoxic regions of tumor mass [8,9].

Metal oxide NPs of zinc (Zn), copper (Cu), silver (Ag), and gold (Au) elements are frequently used in various anti-cancer studies [11-14]. Among all these metal oxide NPs, zinc oxide NPs (ZnO-NP) are considered a promising NP compound due to their selective cytotoxic, biocompatible, easily synthesizable and designable characters, as well as exhibiting anti-microbial, anti-bacterial, anti-oxidant activities [15-17]. With many studies, it has been determined that ZnO-NPs affect different oncogenic intracellular signaling pathways and have antiproliferative effects in many cancer types such as colon, breast, lung, oval, cervical, and stomach at low doses [18-21]. On the other hand, another advantage of ZnO-NPs is that they show their cytotoxic effects on healthy cells at high doses [21]. The selective anti-cancer capabilities of ZnO-NPs are generally thought to proceed by triggering different anti-apoptotic proteins by increasing the production of intracellular reactive oxidant species (ROS) [22-24]. ZnO-NP treatment also depletes levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) enzymes and modulate Bax/Bcl-2 ratio in favor of apoptosis in cancer cells [22]. Also, it was demonstrated that ZnO-NP treatment causes translocation of pro-apoptotic proteins from mitochondria to cytosol [25]. Therefore, one of the main cellular targets of ZnO-NPs is mitochondria, therefore it could be thought that ROS inducing activity of ZnO-NPs must be related with disturbed membrane potential of the mitochondria [22,25].

Enhanced intracellular ROS levels are also indirectly related to cell's energy metabolism, and cancer cells are the cells with disturbed energy metabolism [26]. Cancer cells almost always prefer glycolysis for ATP production because ATP production by oxidative phosphorylation is the main source of intracellular ROS, thus increased amounts of ROS are associated with increased apoptotic stimulus [27]. Therefore, it is conceivable that there may be a mechanistic relation between ZnO-NP and Warburg effect. Evaluation of pyruvate dehydrogenase (PDH) and Lactate dehydrogenase A (LDHA) protein levels are used to test of modulation effects of some candidate modulators on the energy metabolism of cells and enhanced PDH levels are related to enhanced apoptotic stimulation [26-28].

In this study, we aimed to investigate anti-cancer properties and anti-Warburg effects of ZnO-NPs on two breast adenocarcinoma cell lines and HUVEC cells for toxicity assays. MDA-MB-231 and MCF7 cells were used because these cells represent two different breast cancer phenotypes, respectively triple negative (TNBC) and hormone positive (HR+) subtypes. For this aim, the anti-proliferative, anti-colonial, and apoptosis induction and effects of ZnO-NPs on protein levels of PDH and LDHA were investigated.

Material and Methods

Cell Culture

MDA-MB-231 and MCF7 breast adenocarcinoma cell lines (ATCC, USA) and human Umbilical Vein Endothelial Cell (HUVEC) lines (ATCC, USA) were used in the study. Breast cancer cell lines were selected for the different phenotypes they represent; as a TNBC phenotype MDA-MB-231 cell line represent more aggressive and less curable phenotype while MCF7 cell line represent well-curable HR+ phenotype of breast cancer. Cells were cultured in sterile petri dishes with Dulbecco's Modified Eagle Medium (DMEM) (SIGMA, USA) containing 10% fetal bovine serum (FBS) (SIGMA, USA) and 1% antibiotic (Penicillin-Streptomycin) (GIBCO, USA) and incubated in an incubator with a temperature of 37°C, 5% CO_2 and free moisture. All the used cell lines were provided as commercially and an approval of ethical is not necessary.

Cell Viability Assay

Cells were seeded in sterile 96-well microplates containing $3x10^3$/100 µL cells in each well (n=6). The seeded cells were incubated under the specified conditions for 48 h, ensuring ~70% reproduction. Concentrations of 10 µg/mL and 20 µg/mL of ZnO-NP (Nanografi Nano Technology, Türkiye) with a diameter of 30-50 nm were applied to the cells for 24 h, and at the end of the 23rd h, 10 µL of CVDK-8 Cell Viability Kit (EcoTech, Türkiye) was added to each well. A group that was untreated with ZnO-NP was included in the experiment as the control group in each cell lines. The microplate was incubated at 37°C for 60 min and at the end of the 24th h in total, the absorbance values of the samples were obtained by reading at 450 nm via Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). The obtained absorbance values were converted into percentage values using the absorbance values of the control group as 100% and used in statistical analysis.

Doubling Time

When the cells were planted in a 96-well sterile microplate, the initial absorbance values were obtained. By using the initial absorbance values obtained and the absorbance values of the application groups at the end of the 24th h, the

folding times were determined according to the groups. Online Doubling Time Calculator Tool *(http://www.doubling-time.com/compute.php)* was used to determine the folding times for the calculation of folding times.

Cell Harvesting and Homogenization

Cells seeded in sterile petri dishes were treated with 10 µg/mL and 20 µg/mL ZnO-NP doses and incubated for 24 h. At the end of the time, the media were removed and the cells were washed twice with cold PBS (pH: 7.4) on ice, and the cells were scraped with 2 mL of cold PBS and transferred to Eppendorf tubes. The tubes were centrifuged at 4°C and 1200 rpm for 5 min, and pellets were obtained by removing supernatants. Pellets were lysed by a freeze-thaw method using lysis buffer (TNN + NP40 + PMSF). Total protein amounts were determined using the Bradford Protein Assay Kit II kit (Biorad, USA) at a wavelength of 595 nm.

Western Blotting

Samples were standardized to forty µg of protein and subjected to 10% Tris-Glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blotting was carried out using polyvinylidene difluoride membranes (PVDF). The membranes were incubated overnight at 4°C with anti-pyruvate dehydrogenase (PDH) (Cell Signaling, USA), anti-lactate dehydrogenase (LDHA) (Cell Signaling, USA) and anti-β-actin (Santa Cruz, USA) primary antibodies. After three washes, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, UK) at room temperature for 30 min. Subsequently, the membranes were washed, and images were captured following treatment with the enhanced chemiluminescence substrate (ECL) (Biorad, USA).

Colony Formation Assay

Cells were seeded in a sterile 6-well plate containing 200 cells in each well (n=6) and incubated for 48 h. At the end of the period, the cells were treated with 10 µg/mL and 20 µg/mL ZnO-NP and the experiment was continued for 14 days. Also, a group that was untreated with ZnO-NP was included in the experiment as a control group in each cell lines. At the end of the experimental period, the medium was removed, and the wells were washed with PBS (pH: 7.4). After washing, the cells were fixed with fixative buffer (1 acetic acid: 7 ethanol). Following the procedure, the colonies were stained with crystal violet. The plates were placed in a water tank to remove crystal violet remains and the plates were left to dry overnight. The drying plates were photographed, and the colonies were converted into numerical data by using the Analyze Particle Tool in the ImageJ 1.53m program and used in statistical analysis [31]. Colonies containing fewer than 50 cells were not included in the calculation.

Acridine Orange (AO) and Ethidium Bromide (EB) Staining

Cells were seeded in sterile plates with 24 wells at a density of $1.5x10^3$/mL and incubated for 48 h. At the end of the period, the cells were incubated for 24 h with doses of 10 µg/mL and 20 µg/mL ZnO-NP. Also, ZnO-NP untreated groups were considered as control group for each cell lines. After incubation, each well was washed with PBS (pH: 7.4) and stained with a solution containing 100 µg/mL AO and EB. Cell images were photographed via a fluorescence microscope (EVOS FL, Invitrogen) and images taken from red and green channels were merged. For statistical analysis, 200 cells were counted in each group and classified as live, dead, and apoptotic.

Statistical Analyses

Microsoft Excel 365 and IBM SPSS 26.0 were used to perform statistical analyses. Shapiro-Wilk normality test and Levene homogeneity test were applied to the results. A one-Way ANOVA test was applied to the parameters in line with the significance values obtained and the Games-Howell PostHoc test was used for pairwise comparisons. Frequencies evaluated with Pearson Chi-Square (χ^2) test. All quantitative analyses were repeated six times. Results were considered statistically significant at $P<0.05$.

Results

ZnO-NP is Cytotoxic on Breast Cell Lines but Not on HUVEC Cell Line

The effects of 10 µg/mL and 20 µg/mL ZnO-NP treatments in MDA-MB-231, MCF7, and HUVEC cell lines on the survival at the end of 24 h were shown in *Fig. 1*. The results showed that ZnO-NPs have a cytotoxic effect on both cancer cell lines. It was determined that both treatment doses significantly reduced cell survival compared to the control group ($P<0.001$) in MDA-MB-231 cells. However, there was no statistically significant difference between these two dose groups in MDA-MB-231 cells. However, a dramatic decrease in cell survival was observed in the group treated with 20 µg/mL ZnO-NP ($P<0.001$) in MCF7 cells, while no cytotoxic effect was observed in the 10 µg/mL ZnO-NP treated group. On the other hand, no cytotoxic inhibition in cell survival was observed in HUVEC cells for both ZnO-NP treated groups *(Fig. 1-B)*.

Periods of Doubling Times of Breast Cancer Cells Increase with ZnO-NP Treatment

Fig. 2 illustrates the impact on the cell doubling times of MDA-MB-231 and MCF7 breast cancer cell lines following the application of 10 µg/mL and 20 µg/mL ZnO'NPs for 24 h. Findings from the doubling times showed that 10

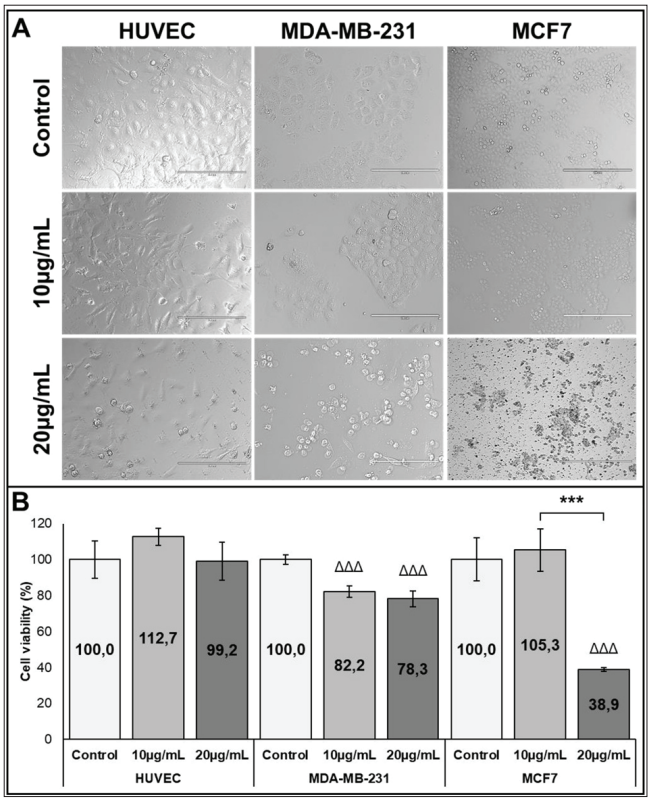

Fig 1. The effects of 10 µg/mL and 20µg/mL ZnO-NPs doses applied for 24 h on cell survival of MDA-MB-231 and MCF7 breast adenocarcinoma cancer cells and HUVEC non-cancerous cells are shown in A (calibration bar=200 µm). The numerical data obtained by converting the absorbances obtained as a result of measurements with the CVDK-8 Cell Viability kit into percentages compared to the control groups and the statistical analysis results are shown in B. The delta (Δ) symbol indicates the statistical difference between the relevant groups compared to the control group, and the star symbol (*) indicates the statistical difference between the specified groups. ($^{\Delta\Delta\Delta}$, ***P<0.001)

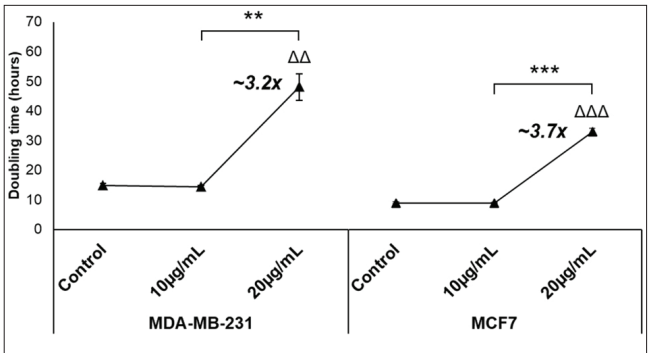

Fig 2. Effect of 10µg/mL and 20 µg/mL ZnO-NPs doses applied to MDA-MB-231 and MCF7 breast adenocarcinoma cancer cell lines for 24 h on cell doubling times. The delta (Δ) symbol indicates the statistical difference of the relevant groups compared to the control group, and the star symbol (*) indicates the statistical difference between the specified groups. ($^{\Delta\Delta}$, **P<0.01, $^{\Delta\Delta\Delta}$, ***P<0.001)

µg/mL ZnO-NP administration had no effect on cell folds, but 20 µg/mL ZnO-NP administration increased the folding times by ~3.2 times (P<0.01) in MDA-MB-231 cells and ~3.7 times (P<0.001) in MCF7 cells.

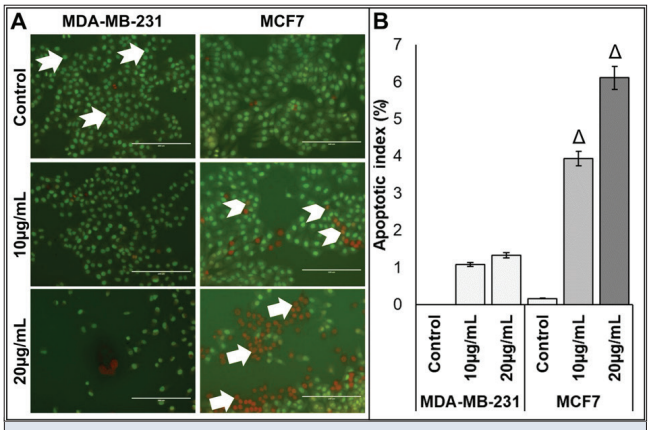

Fig 3. Effects of 10 µg/mL, 20 µg/mL ZnO-NPs doses applied to MDA-MB-231 and MCF7 breast adenocarcinoma cancer cell lines for 24 h on apoptosis, effects by fluorescence microscopy and acridine orange (AO)/ethidium bromide (EB) staining, panel A' is also shown (calibration bar = 200 µm). Cut arrows with a head indicate live cells; cut arrows with no head indicate apoptotic cells; uncut arrows with a head indicate dead cells. Chi-Square (χ^2) statistics test results of apoptotic and dead cell formations are also shown in B ($^{\Delta}$P<0.05)

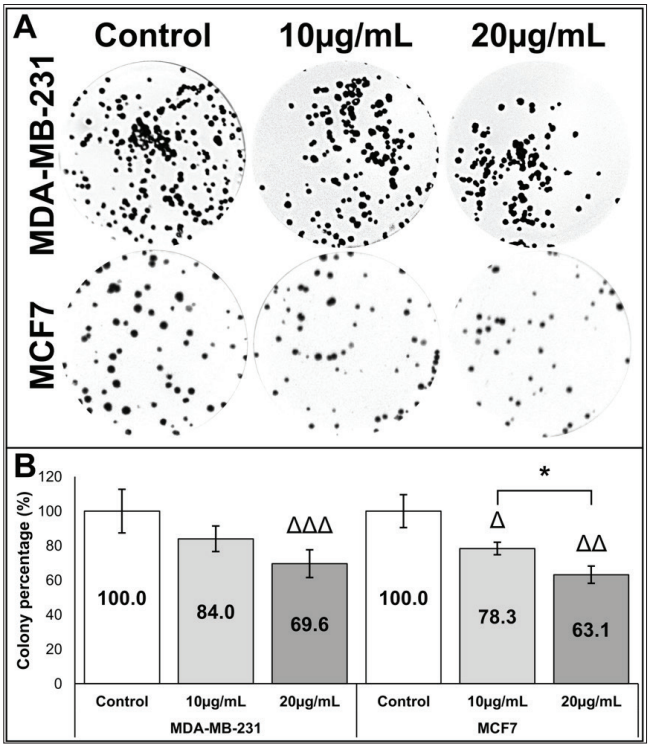

Fig 4. The effects of 10 µg/mL and 20 µg/mL ZnO-NPs doses applied for 14 days on colony formation of MDA-MB-231 and MCF7 breast cancer cells are shown in A. Statistical differences obtained by converting colony numbers into percentage values are shown in B. The delta (Δ) symbol indicates the statistical difference of the relevant groups compared to the control group, and the star symbol (*) indicates the statistical difference between the specified groups ($^{\Delta\Delta\Delta}$, *P<0.001)

ZnO-NP Treatment Induces Apoptotic Index in MCF7 Cell Line

AO stains the nuclei of the living cell green, while EB only stains cells that have lost their membrane integrity

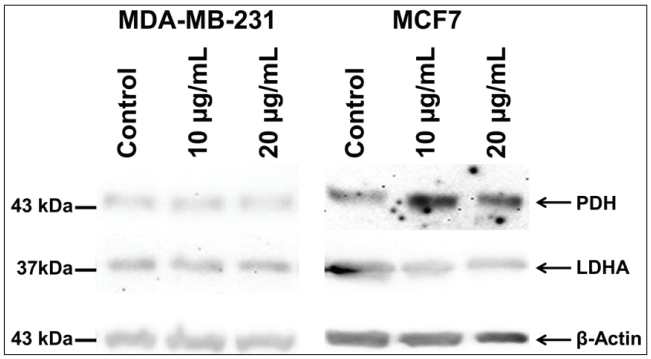

Fig 5. MDA-MB-231 breast cancer cell lines were treated with 10 µg/mL, 20 µg/mL and 40 µg/mL concentrations of ZnO-NPs for 24 h. Samples were standardized to include 40 µg protein in each well. β-actin was used as a loading control

red. For this reason, living cells appear in green, dead cells appear in red, and apoptotic cells appear in orange [32]. Merged AO (green) and EB (red) fluorescent images of both breast cell lines after ZnO-NP treatments for 24 h were shown in *Fig. 3-A*, and frequencies of the apoptotic cells were shown in the graph in *Fig. 3-B*. It was observed that apoptotic formation did not induce in MDA-MB-231 cells treated with ZnO-NP. On the other hand, 10 µg/mL ZnO-NP treatment increased apoptotic index up to ~4 times and 20 µg/mL ZnO-NP treatment increased up to ~6 times ($P<0.05$) in MCF7 cells.

Treatment of ZnO-NP Suppresses Colony Forming Ability of MDA-MB-231 and MCF7 Cell Lines

The colony percentages of MDA-MB-231 and MCF7 breast cancer cells treated with ZnO-NP doses at the end of 14 days were shown in *Fig. 4-A*. 10 µg/mL ZnO-NP treatment reduced colony numbers about ~16% in MDA-MB-231 cells and ~22% in MCF7 cells ($P<0.05$) when compared with the control groups. MDA-MB-231 cells exhibited a reduction of ~31% in their colony-forming abilities compared to the control group when treated with 20 µg/mL ZnO-NP ($P<0.001$). Similarly, MCF7 cells showed suppression of ~37% ($P<0.01$) under the same conditions *(Fig. 4-B)*.

PDH Protein Expression of MCF7 Cells is Enhanced with ZnO-NP Treatment

Expression changes in PDH and LDHA proteins of MCF7 and MDA-MB-231 cells after 24 h of ZnO-NP treatment were shown in *Fig. 5*. β-actin antibody was used as a loading control. Results indicate that neither 10 µg/mL nor 20 µg/mL of ZnO-NP treatment did not affect PDH and LDHA protein expressions in MDA-MB-231 cells. On the other hand, PDH expression of MCF7 cells after ZnO-NP treatments was found as enhanced but LDHA expression was not affected. However, there was no expression difference between 10 µg/mL and 20 µg/mL ZnO-NP treatment groups.

Discussion

ZnO-NPs are metal oxide nanoparticles that selectively induce cell death in cancer cells [33]. ZnO-NPs have been supported by *in vivo* and *in vitro* studies that show several biological effects that can cause cell toxicity, including cytotoxicity, and also inhibit cell proliferation [24,34–36]. Studies have shown that ZnO-NPs have cytotoxic effects on a large cancer cell spectrum such as HepG-2, MCF-7, HeLa, U87, and S91 cells [37]. Similarly, our results show that ZnO-NPs are cytotoxic to MCF7 and MDA-MB-231 breast cancer cells, but MCF7 cells are more sensitive to ZnO-NP treatment. On the other hand, it is known that ZnO-NPs are used in photodynamic therapy methods and as drug transporters due to their low cytotoxicity and targeted selective properties [38-41]. Our findings also show that cytotoxic doses of ZnO-NPs on MCF7 and MDA-MB-231 breast cancer cells do not cause cytotoxic effects on umbilical endothelial HUVEC cells.

The experimental effects of ZnO-NPs are not limited just to *in vitro* studies, there are some promising *in vivo* results for the treatment of cancer. It was shown that ZnO-NP synergistically increased the effectiveness of anti-cancer drugs by inducing apoptosis *in vivo* conditions by Nabil et al.[42]. The disruption of the balance between apoptosis and proliferation in cells is also an important element of carcinogenesis. Therefore, activating apoptotic pathways is a useful anti-cancer strategy. ZnO-NPs are also thought to induce apoptosis by affecting pro-apoptotic proteins in mitochondria, which play key roles in inducing apoptosis [33,43]. The data obtained in this study show that 20 µg/mL dose application to MCF7 cells, which are more sensitive to ZnO-NP treatment, increases the apoptotic index up to 6 times. However, we did not observe the same effect in MDA-MB-231 breast cancer cells. We think that the difference between these two breast cancer cells may be related to the different breast cancer phenotypes (TNBC and HR+) that these cells represent.

Colony formation is the ability of a cell to multiply and form a colony under the stress of division, which is suppressed when left alone. Most of the cancer cells originate from only a single cell and they can metastasize and invade to distant tissues or nearby tissues. Therefore, an effective anti-cancer agent is also expected to suppress the colony-forming ability of cancer cells [44]. NPs are also suppressor agents on the colony-forming ability of cancer cells [24,45]. Our findings also show that ZnO-NPs suppress the long-term colony formation ability of MCF7 and MDA-MB-231 breast cancer cells. However, the dose of ZnO-NP (20 µg/mL), which suppresses cell viability more effectively in MCF7 cells compared to MDA-MB-231 cells, does not show inhibition in the same proportion of its effect on colony formation of MCF7 cells. We think

that this may be related to the rate of degradation of the NP structure in the medium or the resistance developed by cancer cells to cytotoxic ZnO-NP stress in the long term.

Changes in the expression of many proteins in cancer cells cause some conditions. Cancer cells convert pyruvate to lactate, which is formed as a result of glycolysis via Warburg effect. This process gives less energy than glycolysis while providing rapid energy production in cancer cells [46]. For this reason, modulating energy metabolism in cancer cells is a useful anti-cancer strategy. There is some evidence indicating that NPs suppress the Warburg effect. It was demonstrated that AuNPs reduce glycolysis by suppressing the activity of Glucose transporter 1 (GLUT1) and Hexokinase 2 (HK2) enzymes [47]. On the other hand, another study shows that suppressing the Warburg effect via an NP-mediated manner is dependent on induced ROS in osteosarcoma Saos-2 cells [48]. These findings indicate that NPs affect cellular ROS metabolism and cause apoptotic formation. In the present study, we also examined PDH and LDHA protein expression in ZnO-NP-treated breast cancer cell lines MCF7 and MDA-MB-231. LDHA and PDH proteins are two proteins that are closely related to the Warburg effect due to their key role in the balance between glycolysis and oxidative phosphorylation in cancer cells. Our results indicate that ZnO-NP treatment causes an increase in PDH expression but not in LDHA expression in MCF7 cells which represent the HR+ breast cancer subtype however we did not find the same results in MDA-MB-231 cells which represent the TNBC subtype. There are considerable amounts of previous reports indicating that inhibition of PDH is related to promoted apoptotic stimulation [28,29,49]. Therefore, as expected, ZnO-NP-treated MCF7 cells were the groups in which apoptosis was the most promoted in our study. Also, according to a previous report, zinc facilitates the transport of pyruvate into mitochondria and is closely associated with cellular energy status [50]. Therefore, the regulation of PDH by ZnO-NP is not surprising and needs further mechanistic studies and more cancer models to determine the relation at molecular level.

As a result, it was observed that ZnO-NP inhibited cell viability in MCF7 and MDA-MB-231 breast cancer cell lines with no toxic effects in healthy cells. Also, the ability of colony formation of both cell lines was suppressed thanks to ZnO-NP treatment. Correspondingly, the duplication time of both breast cancer cells was found prolonged. However, ZnO-NPs were more cytotoxic on MCF7 cells and increased the apoptotic index more when compared to MDA-MB-231 cells. On the other hand, we determined an enhanced pattern in PDH expression of ZnO-NPs treated MCF7 cells, which was the most apoptotic stimulation determined. Since the increased activity of PDH is associated with increased apoptotic stimulation, we think that the anti-cancer properties of ZnO-NPs in MCF7 cells lie in the activating effects of PDH. For this reason, ZnO-NP is thought to have the potential to inhibit the growth of cancer cells. However, the apoptotic mechanism induced by ZnO-NP appears to induce different results on mutant metabolic elements between MCF7 cell lines representing the HR+ breast cancer phenotype and MDA-MB-231 cell lines representing the TNBC phenotype. Therefore, we think that further studies should elucidate the apoptotic pathway targets of ZnO-NPs in breast cancer cells representing HR+ and TNBC phenotypes.

Declarations

Availability of Data and Materials: All data are presented in the manuscript.

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Research Article

Determination of the Effect of GDF9, BMP15, BMPR1B Gene Polymorphism, and Environmental Factors for Fecundity by Logistic Regression Analysis in Kangal Akkaraman Sheep [1]

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Abstract

Polymorphisms identified on the BMPR1B, BMP15, and GDF9 genes tend to increase multiple birth and ovulation rates in sheep. In the planned study, the productivity records of the flocks (approximately 41.000) collected from 2016 to 2022, belonging to the Public Breeding of Kangal Akkaraman Sheep in Sivas province TAGEM/58KAK2012-08 sub-project of the Public Animal Breeding National Project, were used. Accordingly, the ear numbers of sheep that gave birth to at least two twins (n=96) and at least two singletons (n=96) were determined from 15 different farms relevant records. According to similar feeding characteristics, environmental variables were grouped as location, year-round feeding type, and seasonal feeding type. DNA groups were genotyped by the PCR-RFLP method for BMPR1B (FecB), BMP15 ($FecX^B$, $FecX^G$, $FecX^I$, $FecX^H$) and GDF9 (FecG) alleles. Accordingly, among the 6 SNPs examined, only the GDF9 gene-FecB SNP was determined polymorphic. Genotypic effect (FecB allele) and environmental effect variables (location, year-round feeding type, seasonal feeding type) were also examined with a logistic model. It was determined that the relevant alleles and environmental variables did not have a statistically significant effect on the twinning phenotype. According to the results obtained, it was thought that the genes associated with multiple births in Kangal Akkaraman breed may have different variants specific to the breed. In addition, it is suggested that this character, which is affected by multiple genes, should be included in the planned breeding studies by considering the interaction of environmental variables and determining the variation of the related genes. In this respect, it is concluded that our study will guide the sequencing studies and multivariate analyses to be planned.

Keywords: BMP15, BMPR1B, Fecundity, GDF9, Gene polymorphism, Logistic regression analysis, Sheep

Introduction

The genes involved in controlling fecundity in sheep have been defined as bone morphogenetic protein receptor 1B (BMPR1B) [1], bone morphogenic protein 15 (BMP15) [2,3], and Growth differentiation factor 9 (GDF9) [3]. Polymorphisms identified on reported genes tend to increase multiple birth and ovulation rates in sheep [4]. All these genes belong to the TGF-β superfamily (transforming growth factor beta) [5], which plays an important role in the process of embryo development, ovulation rate, and offspring number. It has been assumed that marker-assisted selection using both genes were guaranteed to increase the number of lambs per litter in ewes and will have significant economic value for sheep breeders. Numerous fertility-related polymorphisms have been identified in various sheep breeds up to this time. However, few polymorphisms have been consistently detected across different breeds. The Booroola gene polymorphism (FecB) was identified as the first major gene for fertility in sheep in 1980. Later studies have shown that ovulation rate and the number of lambs born

per litter can be genetically regulated by a number of different genes, collectively referred to as fecundity (Fec) genes [6,7]. Actually, in a study conducted by Xu et al.[8], many different alleles of the genes examined in different breeds were reported.

The sheep BMPR1B gene has 11 exons and 10 introns on Chromosome 6. Many single nucleotide polymorphisms (SNPs) have been identified for the sheep BMPR1B gene [9], among these, the c.746A>G, c. 864 T>C and c. 1,113 C>A have been associated with sheep reproduction [10,11]. A large study on the nonsynonymous SNP of c.746A>G, which was also examined in our study and characterized as the FecB allele, showed that damage to the BMP system during follicle development increased mean ovulation in Australian Booroola Merino [12], Small-tailed Han sheep [13] and Hu sheep [14]. It has also been reported that this allele has an additive effect on offspring and ovulation rate, but has negative effects on fetal growth and development and body mass during pregnancy [15]. The BMP15 gene (GDF9B, also known as the FecX gene) is on the X chromosome [2] and encodes bone morphogenetic protein 15, which plays an important role in follicular development in sheep [3]. It consists of an intron and 2 exons. BMP15 gene significantly affects fertility [16]. The same allele that causes reproduction in heterozygous Romneys is called the Inverdale allele ($FecX^{I}$). Polymorphism of this gene, identified in various breeds, are called by different names such as $FecX^{I}$, $FecX^{H}$, $FecX^{G}$, $FecX^{B}$, $FecX^{L}$, $FecX^{R}$, $FecX^{Gr}$ and $FecX^{O}$ [17-19]. Sheep with two inactive copies of the BMP15 gene (homozygous animals) have been reported to be infertile [2,3] and display a similar ovarian phenotype. It has been reported that sheep with a single inactive BMP15 gene (heterozygous animals) exhibit increased fertility and an increased ovulation rate and an increased incidence of twin or triplet births [18,20,21]. The GDF9 gene, a member of the Transforming Growth Factor Beta (TGF-β) superfamily, consists of 1 intron and 2 exons located on chromosome 5 in sheep. It is a necessary gene for regular standard follicular development in sheep. It has been reported that there are 8 different polymorphisms (G1-G8) on the gene [22]. Concerning this gene, ovulation rates in sheep are higher in animals with a heterozygous genotype (1.88-1.78) than in animals with a homozygous (1.22-1.16) genotype [22-24].

In addition to the normal genotype of the Akkaraman breed, which constitutes approximately 40% of the sheep raised in Türkiye, the Kangal Akkaraman variety, which is reared in Sivas and Malatya provinces as a local type, was registered as a separate breed with the Communiqué published in the Official Gazette dated 14.08.2012 and numbered 28384 [25]. PCR-RFLP analysis for FecB, $FecX^{G}$, $FecX^{H}$ alleles in the Kangal breed (n: 42), was performed by Karslı et al.[26] but alleles associated with multiple births could not be detected. The average twin birth rate of Kangal breed sheep has been reported as 22% [27].

In the present study, it was aimed to detect the alleles reported to be associated with twinning in the BMPR-1B, BMP15 and GDF9 genes of Kangal Akkaraman breed sheep that have given birth to at least twice twins and at least twice singletons by PCR-RFLP method and to determine the effect of the relevant SNPs on the number of lambs per birth by logistic regression analysis.

Material and Methods

Ethical Statement

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (ERU-HADYEK, Approval no: 21/243-01.12.2021).

Experimental Design and Examination of Animal Data Records

According to the results of the power analysis the number of animals was determined as 192. Taking into account the litter size record of the herds (approximately 41.000) collected from 2016 to 2022, belonging to the Public Breeding of Kangal Akkaraman Sheep in Sivas province of the Public Animal Breeding National Project, the "TAGEM/58KAK2012-08" sub-project, Data records in the relevant project were examined in Excel format. Ewes giving birth to twins at least twice and singletons at least twice were determined by sorting and filtering in Excel according to years, birth type, and mating characteristics. In order to minimize the effect of inbreeding in the selection of animals showing twin and singleton phenotypes, sampling was carried out from 15 different farms in Şarkışla and Gemerek districts. Since the districts where the farms where the samples were collected may show similar environmental (pasture) conditions, they were also classified into two groups as Location 1 (n:99) and Location 2 (n:93) for logistic regression analysis. Information on the feeding type of the farms year-round and according to the months during the year was also obtained from the public hand breeding project. According to this, it was determined that the feeding types of the farms were different in December-February, different in March, and on pasture in April-November. Therefore, farms with similar feeding conditions during the year and throughout the year were categorized into 5 groups. Related information is reported in *Table 1*.

Collection of Blood Samples and DNA Isolation

Blood samples for genetic studies were collected from the *V. jugularis* of the animals into EDTA vacuum tubes. Blood samples were delivered to the laboratory via cold chain and stored at -20°C until DNA isolation. DNA isolation was performed according to the phenol-chloroform-isoamyl

Table 1. *Year-round feeding type group and seasonal feeding type groups*

Location	Year-Round Feeding Type Group	First Seasonal Feeding Type Group (December-February)	Second Seasonal Feeding Type Group (March)
1 (n:99)	Group 1 (n:20)	Barley-wheat-clover (Group 1, n:20)	Barley-wheat-clover (Group 1, n:20)
	Group 2 (n:9)	Barley-wheat-triticale (Group 2, n:9)	Barley-wheat-triticale (Group 2, n:9)
	Group 3 (n:21)	Barley-wheat-grass (Group 3, n:21)	Barley-wheat-grass (Group 3, n:21)
	Group 4 (n:49)	Barley-wheat-grass (Group 3, n:49)	Concentrate feed-wheat-grass (Group 4, n:49)
2 (n:93)	Group 5 (n:93)	Barley-wheat-clover (Group 1, n:93)	Concentrated feed-wheat-clover (Group 5, n:93)

Table 2. *Genes, regions, primers, band sizes list of PCR-RFLP regions*

Gene	Allel	Primers (Forward- Reverse)	Restriction Enzyme	PCR product size, type	Ref.
BMPR1B	FecB	F: CCAGAGGACAATAGCAAAGCAAA R: CAAGATGTTTTCATGCCTCATCAACACGGTC	*Ava*II	Mutant: 160 bp Non-carrier: 190 bp	[6]
BMP15	$FecX^B$	F: GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA R: TTCTTGGGAAACCTGAGCTAGC	*BstDE*I	Wild: 122, 31 bp Mutant:153 bp	[19]
	$FecX^G$	F: CACTGTCTTCTTGTTACTGTATTTCAATGAGAC R: GATGCAATACTGCCTGCTTG	*Hinf*I	Wild type: 112, 29 bp Mutant: 141 bp	
	$FecX^I$	F: GAAGTAACCAGTGTTCCCTCCACCCTTTTCT R: CATGATTGGGAGAATTGAGACC	*Xba*I	Mutant: 124, 30 bp Non-carrier:154 bp	
	$FecX^H$	F: TATTTCAATGACACTCAGAG R: GAGCAATGATCCAGTGATCCCA	*Ah*1I	Mutant: 218, 22 bp Non-carrier: 240 bp	
GDF9	FecG	F: GAAGACTGGTATGGGGAAATG R: CCAATCTGCTCCTACACACCT	*Hha*I	AA: 410, 52 bp GG: 254, 156,52 bp AG: 410, 254, 156, 52 bp	[3]

alcohol method. Quality and quantity determination of DNAs were determined with the help of nanodrops (Shimadzu, Japan).

PCR-RFLP

The 20 µL total reaction volume of the solution used for the PCR consisted of 1 X buffer solution, $MgCl_2$ (2.0 mmol/L), 0.5 U Taq DNA polymerase, dNTP (0.25 mmol/L) and 3 µL DNA (50 ng/µL). The PCR reactions consist of 1 cycle of pre-denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C 30 sec at annealing temperature (annealing temperature specific to each primer), and 30 sec at 72°C for the elongation step. Finally, the process is terminated by waiting at 72°C for 10 min.

The PCR bands length, restriction enzymes and resulting restricted fragment length of the obtained PCR products are presented in *Table 2*. Restriction endonuclease enzyme restriction protocols of the obtained PCR products were carried out according to the manufacturer's protocol. The resulting restriction and PCR products were run through 2% agarose gel electrophoresis and visualized under UV light.

Statistical Analysis

The created logistic regression model;

$$\log\left(\frac{P(Y=1|X}{1-P(Y=1|X)}\right)=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_6X_6$$

where; Y: Birth type (1: having at least two times single births, 0: having had at least two times multiple births), X_1: BMPR1B, X_2: BMP15, X_3: genotypes of GDF9 gene and X_4: Location effect, X_5: Year-round feeding type effect, X_6: Seasonal feeding type effect.

Coefficients of univariate logistic models were calculated using the maximum probability estimation method. Data analysis was performed with the ISLR package in software R 4.1.2 *(https://www.r-project.org/)*. The enter method was used in the applied logistic regression model. The coefficients in the model were obtained using the maximum probability method. Goodness of fit was tested using the chi-square statistic. Univariate logistic regression analysis was used to identify candidate variables and multivariate logistic regression analysis was used to determine the final model. Statistical significance of variables in the multiple

model was tested using the Wald test. The value of the goodness of fit test of the final model was evaluated using the Hosmer Lemeshow test value. Whether the population was in Hardy-Weinberg equilibrium was evaluated using the chi-square statistic. The significance level was taken as P<0.25 in univariate logistic regression models and P<0.05 in multivariate logistic regression models. Chi-square analyses were also performed with phenotype and polymorphic genotype only.

Results

As a result of the PCR analysis, bands reported in the literature belonging to 6 SNPs were obtained in our study. According to the obtained PCR-RFLP Gel-electrophoresis imaging results, the FecG SNP of the GDF9 gene *(Fig. 1)* was found polymorphic, and the restriction images of the other 5 SNPs were found monomorphic. According to these findings, it was determined that $FecX^{B}$ *(Fig. 2)*, $FecX^{G}$ *(Fig. 3)*, $FecX^{I}$ *(Fig. 4)*, $FecX^{H}$ *(Fig. 5)* alleles belonging to the BMP15 gene, and the FecB *(Fig. 6)* allele belonging to the BMPR1B gene examined in all genotypes in Kangal Akkaraman breed sheep was wild type.

Since 5 of the 6 SNPs examined were monomorphic, only the FecB SNP of the GDF9 gene and the location, year-round feeding type effect, seasonal feeding type effect were examined in the logistic regression analysis. In the resulting logistic regression model, the dependent variable was birth type, while the independent variables were evaluated as farm and FecB SNP of the GDF9 gene. In the applied logistic regression model, no significant effect of farm and FecB SNP of GDF9 gene was found on birth type *(Table 3)* (P>0.05).

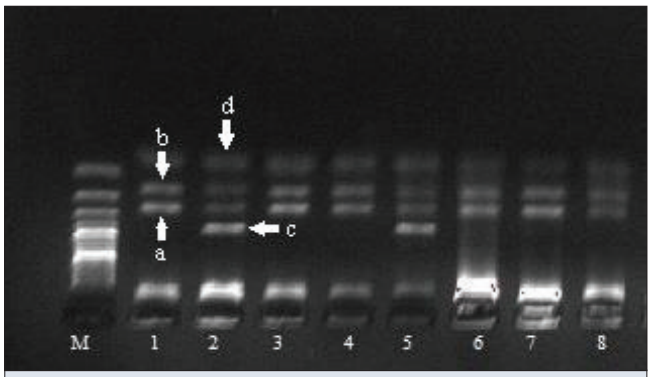

Fig 1. Gel-electrophoresis image of restricted PCR products for GDF9 gene FecG SNP; M: Marker with 100 base pairs; a:254 bp, b: 156 bp, c: 410 bp, d: 52 bp; GG: a, 3, 4, 6, 7, 8 (254, 156, 52 bp) AG: 2, 5 (410, 254, 156, 52 bp)

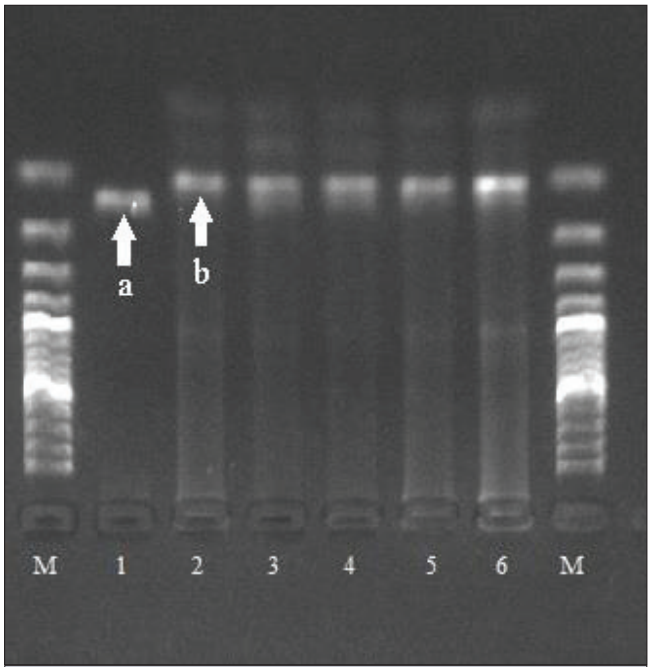

Fig 3. Gel-electrophoresis image of PCR and restricted PCR products for BMP15 gene FecXG SNP; M: Marker with 100 base pairs; a:141 bp, b: 112 bp; PCR product: 1 (141 bp); Wild type RFLP products: 2, 3, 4, 5, 6, 7 (112 bp)

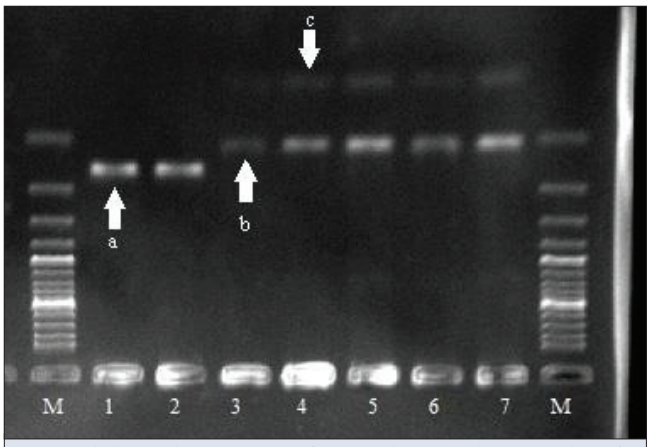

Fig 2. Gel-electrophoresis image of PCR and restricted PCR products for BMP15 gene FecXB SNP; M: Marker with 100 base pairs; a:153 bp, b: 122 bp, c: 31 bp; PCR products: 1, 2 (153 bp); Wild type RFLP products: 3, 4, 5, 6, 7 (122, 31bp)

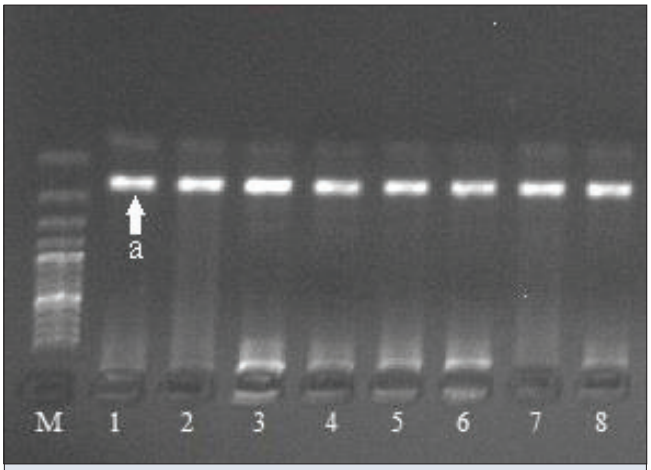

Fig 4. Gel-electrophoresis image of restricted PCR products for BMP15 gene FecXI SNP; M: Marker with 100 base pairs; a:154 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5, 6, 7, 8

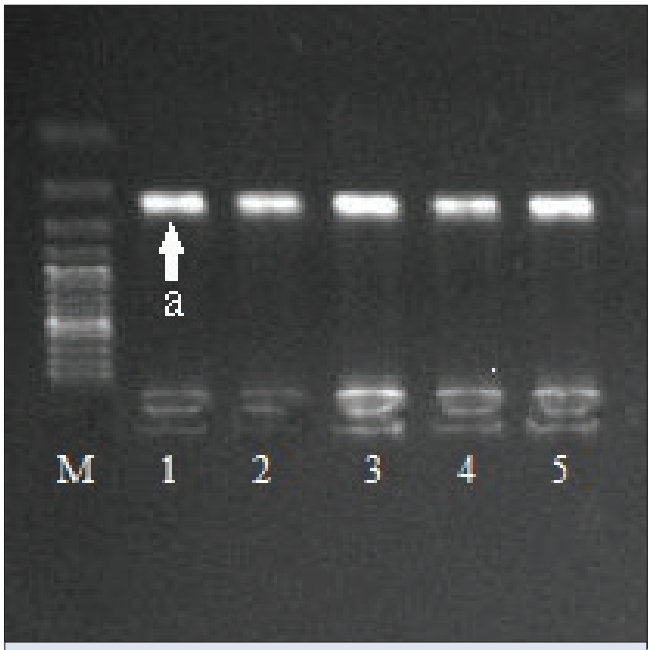

Fig 5. Gel-electrophoresis image of restricted PCR products for BMP15 gene FecXH SNP; M: Marker with 100 base pairs; a:240 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5

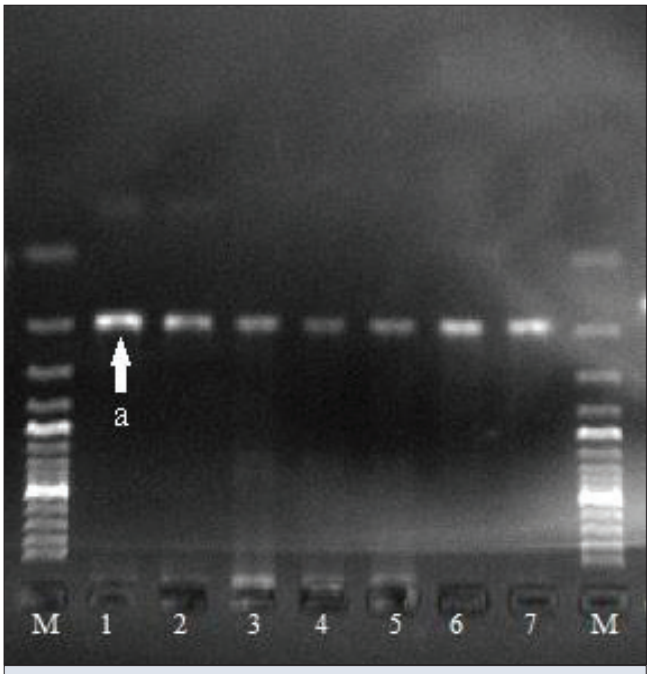

Fig 6. Gel-electrophoresis image of PCR and restricted PCR products for BMPR1B gene FecB SNP; M: Marker with 100 base pairs; a:190 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5, 6, 7

Table 3. *Regression model for birth type and genotype, year-round feeding type, seasonal feeding groups*

Independent Variables	β Coefficient	St Error	Wald Statistic	P Value	Exp(β)	95% CI Exp(β)	
						Upper Limit	Lower Limit
Genotype GG	-0.138	0.346	0.159	0.690	0.871	0.442	1.716
Location 1	-0.432	0.502	0.743	0.389	0.649	0.243	1.735.
FSFTG			1.081	0.582			
FSFTG 1	-0.538	0.540	0.992	0.319	0.584	0.203	1.682
FSFTG 2	-0.367	0.732	0.252	0.616	0.693	0.165	2.905
SSFTG			0.005	0.943			
SSFTG 3	-0.038	0.523	0.005	0.943	0.963	0.345	2.685
Constant	0.592	0.587	1.015	0.314	1.807		

FSFTG: *First seasonal feeding type group,* ***SSFTG:*** *Second seasonal feeding type group*

Table 4. *Correct classification table for birth type*

Observed		Predicted		
		Birth Type		Correct Classification Rate
		Singleton	Twin	
Birth Type	Singleton	72	24	75.0
	Twin	65	31	32.3
Total Correct Classification Rate				53.6

The correct classification rate was found to be 55.2% *(Table 4)*. As a result of the Hosmer and Lemeshow test, the chi-square statistic was found 30.681. Frequencies and Chi-square analysis results for all genes are reported in *Table 5.* For the FecB allele of GDF9 gene, the population was found to be in Hardy-Weinberg equilibrium *(Table 6)* (P=0.073). In addition, sample numbers of locations and phenotype frequencies according to GDF9 genotypes for locations are reported in *Table 7*.

Table 5. *X^2 analysis of phenotype and genotype for each gene allele*

Genes	Genotype	Phenotype		Chi-square P Value
		Twin Observed Count (Expected Count)	Single Observed Count (Expected Count)	
BMPR1B (FecB)	WW	96	96	Monomorphic
BMP15 $FecX^B$	WW	96	96	Monomorphic
BMP15 $FecX^G$	WW	96	96	Monomorphic
BMP15 $FecX^I$	WW	96	96	Monomorphic
BMP15 $FecX^H$	WW	96	96	Monomorphic
GDF9 FecG	AG	23 (22.0)	21 (22.0)	0.118 P=0.731
	GG	73 (74.0)	75 (74.0)	

Table 6. *Hardy-Weinberg equilibrium of the GDF9 gene population*

Gene	Genotypes	Expected	Observed	A Allele	G Allele	Chi-square P Value
GDF9	AA	2.5208	0	0.1146	0.8854	3.2155 P=0.073
	AG	38.9583	44 (0.23)			
	GG	150.5208	148 (0.77)			

Table 7. *Sample numbers of location groups and phenotype frequencies according to GDF9 genotypes*

Location	Phenotype			Genotype		
				AG	GG	Total
Location 1	Phenotype	Twin	Count	12	38	50
			% within genotype	54.5%	49.4%	50.5%
			Count % within phenotype	24.0%	76.0%	100.0%
		Singleton	Count	10	39	49
			% within genotype	45.5%	50.6%	49.5%
			Count % within phenotype	20.4%	79.6%	100.0%
	Total		Count	22	77	101
			% within genotype	100.0%	100.0%	100.0%
			Count % within phenotype	22.2%	77.8%	100.0%
Location 2	Phenotype	Twin	Count	11	35	46
			% within genotype	50.0%	49.3%	49.5%
			Count % within phenotype	23.9%	76.1%	100.0%
		Singleton	Count	11	36	47
			% within genotype	50.0%	50.7%	50.5%
			Count % within phenotype	23.4%	76.6%	100.0%
	Total		Count	22	71	93
			% within genotype	100.0%	100.0%	100.0%
			Count % within phenotype	23.7%	76.3%	100.0%

Discussion

Many studies on DNA regarding sheep fertility and litter size have been reported. The first studies conducted with molecular methods on this character reported that the polymorphism known as the Booroola allele was on exon 8 of the BMPR1B gene. Previous studies reported that the polymorphism of this gene was identified in 48 breeds living in 19 countries [28]. In addition, later studies have also showed the polymorphism of this gene in different breeds [29-31]. On the other hand, in some of the studies, it has been reported that this polymorphism was not

detected in some breeds [32-37]. In a study of Xu et al.[8] on different breeds with high and low offspring fertility, different candidate gene clusters related to offspring size were identified. For example, in the reviewed study, it was reported that BMPR1B, FBN1 and MMP2 gene clusters in the Wadi breed may play a role in variation according to breeds. Pourali et al.[36] reported no FecB polymorphism in Markhoz goats, but there may be new alleles in exon 8 of the relevant gene. Karslı et al.[26] reported that the FecB allele could not be detected in Kangal Akkaraman (n:42) and South Karaman (n:29) sheep. Similarly, in our study, no polymorphism related to the allele examined in the Kangal Akkaraman breed was detected. Therefore, its relationship with offspring productivity could not be determined. It was determined that all samples examined had wild type allele. Therefore, the related gene was not subjected to logistic regression analysis. It was concluded that the related allele was not found in Kangal Akkaraman breed sheep and could not be associated with twinning.

The other two genes investigated with alleles in our study were BMP15, located on the X chromosome, and GDF9 gene, located on the 5th chromosome. In the present study, the $FecX^G$, $FecX^B$, $FecX^I$, $FecX^H$ alleles located in the Exon 2 region of the BMP15 gene and the $FecG^H$ allele reported on the exon 1 region of the GDF9 gene were examined. In the literature studies reviewed polymorphisms in the BMP15 and GDF9 genes were detected in Belclare and Cambridge sheep breeds by Hanrahan et al.[3]. Polymorphisms in the BMP15 ($FecX^I$, $FecX^H$, $FecX^G$, $FecX^B$) and GDF9 ($FecG^H$) genes in Sakız, Kıvırcık, Awassi and İmroz breed sheep were examined by Gürsel et al.[32], and it was reported that the SNPs examined, except for the $FecX^G$ SNP in the BMP15 gene and GDF9 ($FecG^H$), were determined monomorphic. In the study by Mullen et al.[38] examining BMP15 ($FecX^G$, $FecX^B$) and GDF9 ($FecG^H$) in Belclare and Cambridge sheep, it was reported that $FecX^B$ was determined only in hyper productive sheep breeds and $FecG^H$ was determined in the Lleyn breed. Saleh et al.[37] reported that BMP15 $FecX^G$ and GDF9 FeG^H SNPs were detected in Rahmani and Rahmani x Barki cross. Rezaei et al.[39] in which previously reported SNPs in the BMP15 and GDF9 genes in Persian-black sheep were examined, the wild type allele frequency for GDF9 was reported as (+) (75%) and the mutant allele frequency was reported as (-) (25%). The observed frequency for GG, G+, ++ genotypes was reported as 0.05, 0.40, 0.55, respectively. In a study conducted by Kırıkçı [40] in which SNPs in BMP15 and GDF9 genes were investigated in Of sheep living in the Black Sea region, the DNA of the relevant genes was sequenced. A novel SNP (T755C) in the BMP15 gene and five known SNPs in the GDF9 gene (c471C>T (G2), c477 G >A (G3), c721 G>A (G4), c978 A >G (G5) for a total of six SNPs were defined. It was reported that Çepni and Of breeds showed a highly polymorphic structure in the examined genes. Aymaz et al.[41] detected new SNPs in addition to existing SNPs in the BMP15 (in Kıvırcık, Karacabey Merino, Sakız, Gökçeada, Çine Çaparı, İvesi and Karakaçan breeds) and GDF9 gene regions (in Kıvırcık, Karacabey Merino and Sakız breeds). Tong et al.[30] reported a study examining new variants in the promoter region of the GDF9 gene in Mongolian sheep. In addition, in a study conducted on Of sheep breed with a twinning rate of up to 35-40%, it was reported that the genotypes of the FecG1 (GDF9) allele were found to be heterozygous by the PCR-RFLP method [42]. Polley et al.[29] reported that the G1 locus of the GDF9 gene was polymorphic, and two genotypes were detected: mutant (A) and wild type (G) with allele frequencies of 0.18 and 0.82, respectively. Gorlov et al.[22] reported that when the GDF9 G1 and G4 gene regions were examined, the GDF9 gene was found to have a high frequency of G allele and GG genotype in the G1 region, and A allele and AA genotype in the G4 region. Wang et al.[31] sequenced the entire coding region of the GDF9 gene in Luzhong sheep, and reported that g.41768501A>G, g.41768485 G>A polymorphisms in GDF9 and FecB were significantly associated with litter size in Luzhong sheep. In the study conducted by Kırıkçı [43] on the Akkaraman breed with BMP15 ($FecX^G$ and $FecX^I$) and GDF9 (G1 and G4) genes; the relationship between genotypes and the number of offspring were examined (n: 100). GDF9 G1 was reported to be the only polymorphic SNP among the examined genes. It was reported that the frequencies of GA and GG genotypes were 0.26, 0.74, and A and G allele frequencies were 0.13 and 0.87. According to the association analysis, there was no statistically significant difference between the investigated SNPs and offspring size in that study. Consistent with the finding of Kırıkçı [43] obtained in the Akkaraman breed, in our study, the G1 region on the exon 1 region of the GDF9 gene in the Kangal Akkaraman breed was examined and found to be polymorphic. In our study, it was determined that the A allele frequency (mutant) was 0.1146, the G allele frequency was 0.8854, the AG genotype frequency was 0.23 and the GG genotype frequency was 0.77. According to the results of the logistic regression analysis, it was determined that the effect of the genotypes of the GDF9 gene examined in the model, together with the farm effect, on twinning was not statistically significant. According to the results of logistic regression analyses performed in the presence of environmental variables and genotypic variables, it was determined that genotype and other environmental conditions examined did not have a statistically significant effect on the dependent variable of fecundity. It was determined that in-season and year-round feed type changes in the sample examined in this character, which is especially affected by environmental

effects and the effect of multiple genes, were not effective on the twinning phenotype. According to obtained results, the proportion of animals showing twinning phenotype with AG genotype was determined as 22.2% and 23.7% in locations 1 and 2, respectively. The fact that the farms had no effect on the twinning phenotype suggested that sampling was carried out under similar care and feeding conditions and the variation observed in twinning may be due to different genetic variations. By taking samples from different farms, it was aimed to minimise the genetic relationship and to ensure impartiality in the evaluation of the results. It was also concluded that the obtained results when the FecB allele of the GDF9 gene was subjected to Chi-square analysis only with the twin phenotype was not statistically significant *(Table 5)*.

In Cele Black breed sheep, a SNP was detected at position L251P on the Exon 2 region of the BMP15 gene by Niu et al.[44]. This SNP has been shown to be a significant mutation affecting fertility in Cele Black breed sheep. A 3 bp (CTT) deletion was detected in exon 1. In a study performed by Davis et al.[20], no $FecX^{I}$ allele of the BMP15 gene in any of the tested sheep was reported. Karsli et al.[45] examined the sheep breeds of Akkaraman (24 samples), Dağlıç (19 samples), İvesi (19 samples), Tuj (15 samples) and Karakaş (19 samples) bred in Turkey, and they reported that the polymorphisms in the BMP15 gene ($FecX^{G}$, $FecX^{I}$, $FecX^{H}$, $FecX^{B}$) were monomorphic. In a study conducted on Malin and Dorper sheep by Somarny et al.[34], $FecX^{I}$, $FecX^{H}$, $FecX^{B}$ and $FecX^{G}$ polymorphisms were not determined. In the PCR-RFLP study conducted on the Awassi breed (n=88), the $FecX^{I}$ (Inverdale) allele was investigated, but no polymorphism was detected [46]. In a study performed by Karslı et al.[26] to examine the $FecX^{G}$, $FecX^{H}$ alleles in Kangal (n: 42) and South Karaman (29) sheep. Also another study of Karslı and Balcıoğlu [33] to examine the $FecX^{G}$, $FecX^{H}$, $FecX^{I}$, $FecX^{B}$ alleles in Akkaraman (24 samples), Dağlıç (19 samples), İvesi (19 samples), Tuj (15 samples) and Karakaş (19 samples) sheep breeds. In two study, it was reported that the relevant alleles could not be detected. Also, in our study, four alleles of the BMP15 gene were found as monomorphic wild type. This suggests that the analysed allele has no effect on the twinning phenotype in Kangal Akkaraman breed. Ghoreishi et al.[47] examined the BMP15 and GDF9 genes in Markose goats. They reported that two new mutations were discovered in the relevant genes, which were related to the number of offspring. In a study conducted by Çelikeloğlu et al.[48] , BMPR1B (Exon 9, 10, 13a, 13b), BMP15 (Exon 1, Exon 2) and GDF9 (Exon 1 and exon 2) genes in Pırlak sheep were found that relevant regions of all three genes have monomorphic structures. In a study conducted by Çelikeloğlu et al.[49] on BMPR1B, BMP15 and GDF9 genes from Ramlıç and Dağlıç local breeds, it was reported that many SNPs were detected in the sequencing study of the relevant genes in sheep that gave 60 single births and 60 twin births. These authors detected 36, 4 and 11 SNPs in the GDF9, BMPR1B and BMP15 genes in Ramlıç breed and 40, 3 and 11 SNPs in Dağlıç breed. A total of 16 SNPs in the Ramlıç breed and 10 SNPs in the Dağlıç breed were significant for three genes. Ultimately, from the analysis, four SNPs (g.49496G>A, c.1658A>C, c.2037C>T, c.2053C>T) were shown to exist in the BMPR1B gene and one deletion mutation (c.28-30delCTT) in the BMP15 gene. These authors determined five SNPs (c.1487C>A, c.2492C>T, c.2523G>A, c.2880A>G and c.2763G>A) of the BMPR1B gene of the Dağlıç breed as well as the Ramlıç breed. They suggested that the observed polymorphisms have the potential to be used as genetic markers in the selection of productive animals for both breeds.

It is of great importance to characterize the birth type characteristics that will contribute economically to our country's domestic sheep assets. According to all these literature studies and the results obtained from the present study, it can be seen that the variations of the genes examined vary in each breed. Accordingly, among the 6 SNPs examined in our study, only the GDF9 gene-FecB SNP was determined polymorphic. Genotypic effect (FecB allele) and environmental effect variables (location, year-round feeding type, seasonal feeding type) were also examined with logistic model. It was determined that the relevant alleles and environmental variables did not have a statistically significant effect on the twinning phenotype. According to the obtained results, it was thought that the genes associated with multiple births in Kangal Akkaraman breed may have different variants specific to the breed. In addition, it is suggested that this character, which is affected by multiple genes, should be included in the planned breeding studies by considering the interaction of environmental variables and determining the variation of the related genes. In this respect, it is concluded that our study will guide the sequencing studies and multivariate analyses to be planned.

Declarations

Availability of Data and Materials: The dataset used in the study is available from the corresponding author (E.G. Aksel) on reasonable request.

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Ethical Statement: This study was approved by the Erciyes University

Animal Experiments Local Ethics Committee (ERU-HADYEK, Approval no: 21/243-01.12.2021).

Competing of Interest: All other authors declare no competing of interest.

Author Contributions: EGA, EÇG, MA, ÖOD contributed to the conceptualization, design, funding and supervision of the study. EGA conducted all experiments and wrote the first draft of the manuscript. MA, EÇG, ÖOD 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

The Concentrations of Selective Endocrine Disruptors in Milk from Different Lactation Periods of Cows

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Abstract

Milk can be contaminated with organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and polycyclic aromatic hydrocarbons (PAHs), which are known as endocrine disruptors (EDCs). However, the relationship between the lactation period of cows and the residues of EDCs is unknown. The aim of this study was to determine the relationship between the lactation period in cows and EDCs residues such as OCPs, PCBs, PBDEs, and PAHs. Milk collected from cows during each lactation period was analyzed in terms of fat content and EDC residues. One or more contaminants were detected in almost all (98%) of the milk taken in three lactation periods. Three PCBs and 11 PAHs were found together in 1st lactation period, and one more PAH was added to them in other periods. However, the sample rate exceeded the maximum residue limit of 48%, 10%, and 16% in the first, second and third lactation periods, respectively. Also, it was seen that the riskiest period was the first lactation period, followed by the third and second periods. The same ranking is valid for the fat content in milk. Thus, it was concluded that the excretion of lipophilic EDCs in milk is related to the fat content in milk, and the higher the fat content in milk, the more lipophilic EDCs are excreted in milk.

Keywords: Endocrine disruptor, Lactation period, Milking, Persistent organic pollutants, Polycyclic aromatic hydrocarbons, Risk assessment

Introduction

Persistent organic pollutants (POPs) are substances highly stable to chemical and biological degradation, non-degradable for a long time, and are well soluble in lipids [1]. Organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) are environmental pollutants. OCPs have been widely used in pest control for the plant protection products and protection of public health [2]. PCBs have been used greatly as insulator and cooling liquid, especially in capacitors, transformers, hydraulic pumps, in printing inks and paint production as plasticizer, in carbonless copy papers, in polyvinyl chloride (PVC) coatings of electrical cables to provide durability, etc. [1]. PBDEs are used as flame retardants in diverse products, including building materials, electronic tools, furnishing, motor instruments, airplane, polyurethane foams, and textiles [3].

POPs can lead to various kinds of cancer, anomalies, developmental and reproductive damage, nervous system disturbance, suppression of the immune system, and other harmful effects in humans and animals because of their endocrine-disrupting effects [4]. Thus, since 2001, many countries such as Turkiye, which have evaluated the risks of POPs to the environment and human health, have signed the International Stockholm Convention on POPs to terminate or restrict the production and use of

OCPs and PCBs [1]. The same precautions began to be implemented in the production and use of PBDEs in 2009 [5]. Polycyclic aromatic hydrocarbons (PAHs) are also common environmental pollutants generated during deficient burning of organic matter and anthropogenic movements [6]. Since PAHs are disintegrated easier than other environmental pollutants, they are not usually accepted as POPs. However, they are candidate substances for POPs due to being highly extensive and lipophilic [7].

The milk and dairy products may be contaminated with contaminants such as endocrine disruptors and therefore may both threaten human health and affect the commercial image by creating a technological risk factor [8-12]. One study showed that carryover rates of OCPs from feed to cow milk can range from 0.10% to 250% [13].

Many studies focused on POP residues in milk and risk assessment, however, it is known that the lactation period is an important factor that significantly affects the excretion of xenobiotics [14]. Lactation in cows is divided into three periods. The first period is the beginning of lactation and includes 10 weeks after birth (approximately 70 days). Body reserves are used for milk production. During this period, milk yield increases rapidly and within 6-8 weeks, milk yield reaches its highest value and the animal's feed consumption cannot meet the energy excreted from the body with milk. Thus, the animal uses the fat in its body to meet its energy needs. The second period is between the 70th and 140th days of lactation, and in this period, milk yield and milk fat ratio begin to decrease. The third period covers the 140th to 305th day of lactation. During this period, the animal's feed consumption begins exceeding its needs and the animal regains its body reserves for the next lactation (21-44 weeks). Milk yield had started to decrease. The animal is pregnant and the needs can be easily met as the consumption exceeds the needs [15]. Here, it can be predicted that more POP residues may be encountered in the lactation period when the amount of fat in the milk increases. Because milk is an indicator of the bioconcentration process of POPs, because of their lipophilic properties, they are first stored in fat-rich tissues and then excreted via milk fat [16]. To test this hypothesis, a farm in Ankara was selected and milk was collected from cows according to the lactation period. Thus, it was aimed to establish a relationship between fat content, milk yield and POP residues in cow's milk collected according to the lactation period.

Material and Methods

Ethical Approval

Since only milking was performed in this study, there is no requirement for ethics committee approval according to national legislation. Additionally, informed consent was obtained from the farm owners

Sampling Strategy

A herd of approximately 500 cows in Ankara was selected to test our hypothesis. There were Holstein and Holstein's crossbred cows aged 3-8 years on the farm where milking was done automatically and good veterinary practices were implemented. The lactation period of the cows is approximately 305 days and it has been stated that the milk yield of each cow is between 5.000 and 7.000 liters per year. The cows selected for sampling were in the same environment. During each lactation period, 100 mL-milk, approximately 25 mL from each udder lobe, was milked from 50 cows and mixed. To ensure the results, morning milking was performed on days 49, 50, and 51 in the first lactation period, on days 99, 100 and 101 in the second lactation period, on days 199, 200, and 201 in the third lactation period. All milk samples were taken in a glass jar, brought to the laboratory within 2–3 hours under cold chain, and kept at -20°C until analysis. The analyses were performed no later than 5-7 days after all the samples were collected. Results representing each lactation period were given as the mean of 3 days of milk. Thus, 450 milk samples were analyzed in terms of milk fat content and POP residue. Calculations were made based on the results from 150 cows.

Chemicals and Reagents

Analytical standards for OCPs, PCBs and PAHs were purchased from Dr. Ehrenstorfer (Augsburg, Germany), while those for PBDEs were obtained from Wellington Laboratories (Guelph, Canada). Solvents of analytical grade, sodium chloride (NaCl), and anhydrous sodium sulfate ($MgSO_4$) were procured from Sigma (Steinheim, Germany), while primary-secondary amine (PSA) and C18 Solid Phase Extraction (SPE) adsorbent were obtained from Agilent (Santa Clara, USA). All stock and working solutions were made ready in acetonitrile.

The SPE column was designed by adding 0.2 g C18, 0.4 g PSA, and 0.2 g $MgSO_4$ to the glass Pasteur pipette with glass cotton at the bottom, respectively [1].

Extraction

To extract target EDCs from milk, we used a modified method developed by Simsek et al.[1]. Two g of milk sample was weighed separately in screwed glass tubes with glass beads in it, and was homogenized for 15 min by adding 5-mL acetonitrile, and kept in an ultrasonic bath at room temperature for 10 min. One g NaCl was put in the specimens moved away from the bath and vortexed for 5 min. It was centrifuged at 1968 × g for 10 min at +4°C. The supernatant was taken and transferred to a glass tube, and 3-mL acetonitrile was added to the precipitate left in the tube and shaken in vortex for another 10 min. One g $MgSO_4$ was added to the tube containing the supernatants

and shaken manually for 30 sec and kept in the freezer at -20°C for 12 h. The next day, it was removed from the freezer and centrifuged at 1968 × g for another 10 min. The supernatant was removed and evaporated under nitrogen at 35°C until 1 mL. It was passed through the SPE column previously conditioned with 4×1 mL acetonitrile. The filtrate was collected with 4×1 mL acetonitrile and completely evaporated under nitrogen. The dry residue was collected with 90 µL acetonitrile, and applied to the gas chromatography-mass spectrometry (GC-MS) device by adding 10 µL of PCB30 solution (1‰ w/v). PCB30 is used as an injection internal standard.

Gas Chromatographic Analyses

Analysis of target analytes was done by GC-MS (Thermo Finnigan, CA, USA). The instrument was employed in a splitless mode. Electronic ionization, external ion source, interface and injector port temperatures were set to 70 eV, 250°C, 270°C and 280°C, respectively. In this work, 2 µL sample was applied to the autoinjector. The separation was performed with a HP-5MS capillary column (30 m × 0.25 mm diameter, 0.25 µm film thickness) (Agilent Technologies, CA, USA). Helium (99.995% purity) was used as the carrier gas at a running flow rate of 1 mL/min. A separate furnace work schedule was applied for OCPs, PCBs and PBDEs, and PAHs. The furnace program used for each group was determined according to the method of Simsek et al.[1]. Mass spectrometric evaluation was performed in the selected ion monitoring (SIM) mode using 2 or 3 characteristic fragment ions for each compound (1 target ion and 1 or 2 qualifier ions) *(Table 1)*.

Quality Assurance and Quality Control

The method validation was performed by SANTE guideline [17]. Milk samples without target analytes were used to validation the method. For validation, linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity, precision and recovery parameters were assessed.

The milk fat content was measured by the Gerber method with the help of a butyrometer [18].

Risk Assessment

The Environmental Protection Agency (USEPA) guidelines were followed in calculating the risks to human health of contaminants detected in milk [11]. Risks were determined for both children and adults. Risk assessment was performed based on both the average concentration and the maximum concentration. For this, the estimated daily milk intake (EDI) was calculated first. The EDI depends on both the concentration of each compound and the daily consumption of the food. The following formula was used to calculate the EDI;

$$EDI\ (\mu g/kg/day) = CR \times C/body\ weight$$

where "CR" is the daily milk consumption rate (kg/day), "C" is the concentration of the pollutant measured in milk (µg/kg). According to the Turkiye Nutrition and Health Survey [19], the average daily consumption of milk and dairy products for adults over the age of 15 is 0.19 kg and the body weight is 75 kg. Since milk consumption and body weights are not given to 10-15-year-old children in this guide, a study by Bıyıklı and Akman [20] was used. Accordingly, the average milk consumption of 10-15-year-old children in Türkiye was calculated as 0.3 kg/day and body weight as 33 kg. According to USEPA standards, the risk of non-carcinogenic effects is expressed by comparing the exposure dose with the dose considered to have no effect [21]. This rate is called the target hazardous quotient (THQ) and is calculated as follows, based on the acceptable daily intake (ADI) of each compound.

$$THQ=EDI/ADI$$

If the THQ value is less than 1, exposed individuals do not show significant toxic effects. If one exceeds and the THQ value increases, significant toxic effects can be experienced.

The Hazard Index (HI) is a measure of the risk of potential adverse health effects from the mixture of chemicals in milk [22]. The sum of the THQ values gives the hazard index (HI), which is the sum of the contaminants in the milk samples, and is calculated as follows [9].

$$HI= THQ_1+THQ_2+\ldots. THQ_n$$

The Toxic Equivalency Quotients (TEQ)

We assessed TEQ concentrations of 16 target PAHs with the following formula. The toxic equivalency factors (TEFs) advanced by Nisbet and Lagoy [23] were employed to assess the TEQ_{BaP} value.

$$TEQ_{BaP}=C_i \times TEF_i$$

where, "C_i" is a PAH congener, "i" is the sample concentration (µg/kg), "TEF_i"is the BaP value relative to the potency value published for each individual PAH.

Statistical Analysis

Descriptive statistics were performed on all samples accumulated from different lactation periods. The concentrations of compounds in milk were shown by arithmetic means with minimum and maximum values and standard error (SEM). All statistical analyses were carried out by Stata 12/MP4 statistical software (StataCorp LP, College Station, TX, USA). Duncan multi-scope test was used to compare mean of pollutants different lactation periods. Non detected data were presumed to be half the method limit of quantification for calculation of P value. A significant level of 0.05 was performed for all analyses.

Table 1. *Retention times and selected ion monitoring (SIM) ions for targeted organochlorine pesticides, polychlorinated biphenyls, polybrominated biphenyl ethers and polycyclic aromatic hydrocarbons*

Compounds	Retention Time (min)	SIM Ions	Compounds	Retention Time (min)	SIM Ions
Organochlorine pesticides (OCPs)			***Polycyclic aromatic hydrocarbons (PAHs)***		
α- HCH	12.17	181* 183 219	Naphthalene	5.97	128* 129 102
HCB	12.31	284* 286 282	Acenaphthylene	8.64	150* 153 152
Lindane	13.00	183* 181 219	Acenaphtene	9.07	154* 153
β- HCH	13.24	183* 181 219	Fluorene	10.58	166* 165 163
Heptachlor	15.76	272* 270 274	Phenanthrene	13.85	178* 152 176
Aldrin	17.58	263* 293	Anthracene	14.07	178* 176 152
α-Endosulfan	20.16	251* 253 183	Fluoranthene	19.86	202* 200 203
Dieldrin	22.45	239* 237 235	Pyrene	21.38	202* 200 201
p,p′-DDE	23.04	246* 248 318	Benzo(a)anthracene	29.34	228* 226 229
p,p′-DDD	25.20	235* 165 237	Chrysene	29.34	228* 226
o,p'- DDT	25.47	235* 165 237	Benzo(b)fluoranthene	34.44	252* 253
p,p′-DDT	27.34	235* 165 237	Benzo(k)fluoranthene	34.60	252* 253
Methoxychlor	29.68	227* 274	Benzo(a)pyrene	35.96	252* 253
Polychlorinated Biphenyls (PCBs)			Indeno(1,2,3,c,d)pyrene	41.20	276* 277
PCB28	15.43	186* 258 256	Dibenz(a,h)anthracene	41.47	278* 279
PCB52	16.64	220* 257 292	Benzo(g,h,i)perylene	42.29	276* 277
PCB101	21.36	254* 326 328	***Internal Standards for PAHs***		
PCB118	24.82	326* 256 254	Naphthalene d_8	5.92	108* 136
PCB153	25.84	290* 360 288	Acenaphthene d_{10}	9.02	164* 162
PCB138	27.01	290* 360 288	Phenanthrene d_{10}	13.75	188* 189
PCB180	30.13	324* 396 394	Chrysene d_{12}	29.19	240* 236
Polybrominated Diphenyl Ethers (PBDEs)			Perylene d_{12}	36.24	264* 260
PBDE 28	25.17	246* 248 406			
PBDE 47	30.36	486* 488 484			
PBDE 66	31.11	326* 486 484			
PBDE 85	36.63	404* 406 566			
PBDE 99	34.67	404* 406 566			
PBDE 100	33.58	404* 406 566			
PBDE 153	38.52	484* 486 482			
PBDE 154	37.26	484* 486 482			
PBDE 183	42.95	564* 562 566			
Internal Standards for OCPs, PCBs and PBDEs					
PCB30[a]	13.12	186* 258 256			
PCB153-C13	25.84	372* 302 374			
PCB209	36.10	498* 428 500			

** Quantifier ion; [a] Internal injection standard;* ***α-HCH:*** *alpha- Hexachlorocyclohexane;* ***HCB:*** *hexachlorobenzene;* ***β-HCH:*** *beta-hexachlorocyclohexane;* ***p,p′-DDE:*** *p,p′-dichlorodiphenyl dichloroethylen;* ***p,p′-DDD:*** *p,p′-dichlorodiphenyl dichloroethan;* ***o,p'- DDT:*** *o,p'-dichlorodiphenyl trichlorethane; p,p′-DDT: p,p′-dichlorodiphenyl trichlorethane*

Results

The average milk yield of 305 days in the cows included in the study was measured as 6146.32 kg/cow. While the milk fat content in the first period of lactation was 5.32%, it was 4.13% and 4.68% in the second and third periods, respectively. While average daily milk yield was 21.62 kg in the first period of lactation, it increased in the second period (average 26.11 kg) and decreased in the third period (average 25.46 kg).

The validation data of the method used to determine the contaminants in milk are shown in *Table 2* and *Table 3*. Mean recovery values were found to be between 88.7-105.4%, 90.8-96.9%, 86.5-96.2%, and 88.7-106.8% for OCPs, PCBs, PBDEs and PAHs, respectively, and repeatability and intermediate precision (RSD%) <13.2%

for all analytes. The LOQ values of the method in milk were determined as 0.82-2.28 µg/kg, 1.01-1.98 µg/kg, 0.37-0.92 µg/kg and 0.33-0.63 µg/kg for OCPs, PCBs, PBDEs and PAHs, respectively.

One or more contaminants were found in almost all milk samples (98%) during all lactation periods *(Table 4)*. For example, PCB28, PCB153, PCB180, *p,p'*-DDE, phenanthrene, anthracene, fluoranthene, pyrene, benz(a) anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3,c,d)pyrene, dibenz(a,h) anthracene and benzo(g,h,i)perylene were found together in the first lactation period, and acenaphthene was added to them in the second and third lactation periods.

PAH4 and benzo(a)pyrene were predominantly detected during the second lactation period. PAH4 was detected the least during the first lactation period, whereas benzo(a) pyrene was detected the least during the third lactation period. PAH16 and PAH8 groups are the most common compounds in all lactation periods with 98% and 74%, respectively *(Table 5)*.

The highest total indicator PCBs and *p,p'*-DDE

Table 2. *Validation data of targeted organochlorine pesticides, polychlorinated biphenyls and polybrominated biphenyl ethers in cow milk*

Compound	Linearity (µg/kg)	Correlation Coefficient (r^2)	LOD (µg/kg)	LOQ (µg/kg)	Mean Recovery (%)	Repeatability (RSD%)	Intermediate Precision (RSD%)
Organochlorine pesticides (OCP)							
α- HCH	1-100	0.990	0.73	2.2	93.2±10.1	3.0	8.1
β- HCH	1-100	0.995	0.63	1.89	101.3±11.4	9.4	6.3
Lindane	1-100	0.991	0.68	2.05	105.4±9.5	7.7	9.7
HCB	1-100	0.997	0.51	1.54	95.6±7.8	2.3	8.6
HEP	1-100	0.995	0.45	1.36	96,4±9.4	10.1	7.3
α-Endosulfan	1-100	0.997	0.51	1.53	89.8±9.9	10.8	5.1
Aldrin	1-100	0.995	0.77	2.3	92.4±11.2	7.6	10.4
Dieldrin	1-100	0.995	0.76	2.28	88.7±10.1	11.0	9.9
p,p'-DDD	1-100	0.995	0.43	1.29	106.8±5.7	8.3	9.3
p,p'-DDE	1-100	0.998	0.31	0.94	97.9±6.5	2.1	6.8
o,p'- DDT	1-100	0.995	0.43	1.29	93.6±6.5	6.7	8.4
p,p'-DDT	1-100	0.999	0.27	0.82	95.4±9.2	7.2	6.8
Methoxychlor	1-100	0.998	0.54	1.62	97.4±8.4	9.9	7.3
Polychlorinated Biphenyls (PCBs)							
PCB28	1-100	0.993	0.44	1.33	93.6±6.5	8.6	2.2
PCB52	1-100	0.992	0.43	1.28	95.4±9.2	7.3	10.3
PCB101	1-100	0.994	0.48	1.45	95.6±7.7	5.1	9.7
PCB118	1-100	0.993	0.49	1.47	93.9±7.1	4.9	4.8
PCB153	1-100	0.997	0.34	1.01	90.8±8.2	2.2	13.2
PCB138	1-100	0.995	0.36	1.07	95.0±5.9	10.1	3.6
PCB180	1-100	0.991	0.66	1.98	96.9±9.3	6.8	5.3
Polybrominated Diphenyl Ethers (PBDEs)							
PBDE 28	1-100	0.993	0.12	0.37	93.3±3.8	4.9	8.1
PBDE 47	1-100	0.997	0.18	0.53	93.8±5.8	5.5	6.3
PBDE 66	1-100	0.994	0,20	0.61	96.2±6.9	7.3	9.7
PBDE 99	1-100	0.995	0.20	0.59	91.2±7.2	6.2	8.6
PBDE 85	1-100	0.993	0.16	0.47	92.5±3.9	4.8	7.3
PBDE 100	1-100	0,994	0.16	0.49	90.1±8.4	3.6	5.1
PBDE 153	1-100	0.991	0.17	0.52	92.5±3.9	5.3	4.9
PBDE 154	1-100	0.992	0.30	0.89	91.1±1.7	2.2	2.2
PBDE 183	1-100	0.990	0.31	0.92	86.5±8.3	10.1	10.1

Table 3. *Validation data of targeted polycyclic aromatic hydrocarbons in cow milk*

Compound	Linearity (µg/kg)	Correlation Coefficient (r^2)	LOD (µg/kg)	LOQ (µg/kg)	Mean Recovery (%)	Repeatability (RSD%)	Intermediate Precision (RSD%)
Naphthalene	1-100	0.988	0.21	0.63	88.7±10.1	3.0	9.4
Acenaphthylene	1-100	0.992	0.17	0.51	106.8±5.7	9.4	10.5
Acenaphthene	1-100	0.991	0.19	0.57	97.9±6.5	7.7	12.3
Fluorene	1-100	0.995	0.14	0.42	90.9±8.2	2.3	3.7
Phenanthrene	1-100	0.995	0.19	0.57	95.0±5.9	10.1	5.8
Anthracene	1-100	0.994	0.17	0.51	95.1±6.9	10.8	5.8
Fluoranthene	1-100	0.996	0.18	0.54	91.2±7.2	7.6	10.1
Pyrene	1-100	0.996	0.15	0.45	92.3±6.4	11.0	12.9
Benzo(a)anthracene	1-100	0.994	0.11	0.33	97.0±6.3	8.3	7.6
Chrysene	1-100	0.993	0.12	0.36	93.0±6.7	2.1	3.2
Benzo(b)fluoranthene	1-100	0.996	0.15	0.45	90.5±7.2	6.7	4.8
Benzo(k)fluoranthene	1-100	0.997	0.15	0.45	95.1±6.9	7.2	4.8
Benzo(a)pyrene	1-100	0.997	0.12	0.36	91.2±7.2	9.9	3.6
Indeno(1,2,3,c,d)pyrene	1-100	0.994	0.15	0.45	95.1±6.9	2.3	5.3
Dibenz(a,h)anthracene	1-100	0.992	0.13	0.39	91.2±7.2	4.9	2.2
Benzo(g,h,i)perylene	1-100	0.994	0.17	0.51	92.3±6.4	4.9	8.1

Table 4. *Frequency of milks with and without detected endocrine disruptor residues, and milks containing residues above maximum residue level (MRL) for milks collected during different lactation periods*

Lactation Period	No Residue Detected Sample Rate (%)	Sample Rate <MRL (%)	Sample rate >MRL (%)
First	2	50	48
Second	2	70	28
Third	2	60	38

Table 5*. Detected endocrine disruptors residues in milks collected during different lactation periods*

Compound	MRL (µg/kg)	1. Lactation Period		2. Lactation Period		3. Lactation Period		References for MRL
		D.R. (%)	>MRL (%)	D.R. (%)	>MRL (%)	D.R. (%)	>MRL (%)	
Total indicator PCBs*	40	56	10	56	8	56	12	[24,25]
Benzo(a)pyrene	1	18^{b}	4	22^{a}	0	16^{bc}	0	[24,25]
PAH4	1	50^{bc}	30	54^{a}	18	52^{ab}	22	[24,25]
PAH8	n.a.	74	n.a	74	n.a.	74	n.a.	-
PAH16	n.a.	98	n.a.	98	n.a.	98	n.a.	-
p,p'-DDE	20	28	4	28	2	28	4	[26]

** Total indicator PCBs are PCB28, PCB138, PCB153, and PCB180;* ***D.R.:*** *Detection rate;* ***n.a.:*** *Not applicable*
^{abc} *Differences between means with different letters in the same row are significant (P<0.05)*
PAH4: *Benzo(a)piren, benzo(a)anthrasen, benzo(b)fluoranthene and chrysene;* ***PAH8:*** *PAH4 + Benzo(k)fluoranthene, Benzo(g,h,i)perylene, Dibenz(a,h)anthracene and Indeno(1,2,3,c,d) pyrene.*

concentrations were observed in the first lactation period (19.91±10.1 and 2.74±1.32 µg/kg, respectively), followed by the third (17.93±8.9 and 2.35±1.6 µg/kg, respectively) and second lactation periods (15.79±7.93 and 2.2±1.3 µg/kg, respectively). Whereas, the highest total PAH concentrations (PAH8 and PAH16) were seen in the third lactation period (5.60±3.2 and 13.01±7.3 µg/kg, respectively), followed by the first (1.22±0.8 and 0.93±0.1 µg/kg, respectively) and second lactation periods (2.73±1.1 and 9.81±3.9 µg/kg, respectively) (*Table 6*).

Table 6. Mean concentrations of detected endocrine disruptors in milks collected during different lactation periods. (µg/kg) (Mean±SEM)

Compound	1. Lactation Period		2. Lactation Period		3. Lactation Period	
	Range	Mean	Range	Mean	Range	Mean
Milk fat content	5.16-5.56	5.32%[a]	3.99-4.29	4.13%[bc]	4.52-4.87	4.68%[b]
Total indicator PCBs	<LOQ-125.63	19.91±10.1[a]	<LOQ-98.26	15.79±7.93[bc]	<LOQ-112.27	17.93±8.9[ab]
Benzo(a)pyrene	<LOQ-1.12	0.22±0.12	<LOQ-0.75	0.21±0.1	<LOQ-0.85	0.20±0.1
PAH4	<LOQ-8.24	1.22±0.8[a]	<LOQ-2.73	0.93±0.1[b]	<LOQ-3.18	0.93±0.1[b]
PAH8	<LOQ-11.41	3.43±1.54[b]	<LOQ-7.74	2.73±1.1[c]	<LOQ-21.85	5.60±3.2[a]
PAH16	<LOQ-29.76	11.19±7.6[b]	<LOQ-24.73	9.81±3.9[c]	<LOQ-32.11	13.01±7.3[a]
p,p'-DDE	<LOQ-32.03	2.74±1.3[a]	<LOQ-24.36	2.2±1.3[bc]	<LOQ-26.35	2.35±1.6b

***LOQ:** Limit of quantification; [abc] Differences between means with different letters in the same row are significant (P<0.05)*

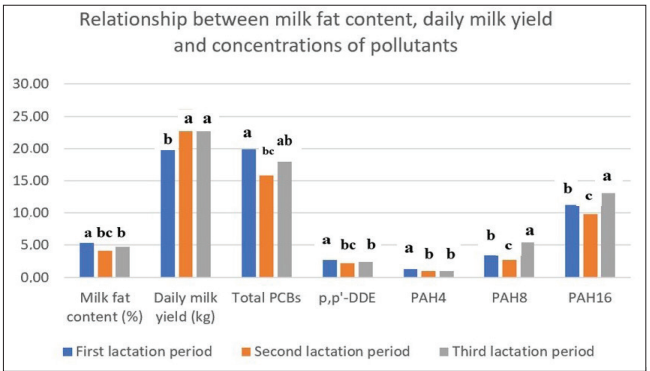

Fig 1. Relationship between milk fat content, daily milk yield and mean concentrations of endocrine disruptors (P>0.05)

However, the highest concentrations of total PCB, PAH4, and *p,p'*-DDE were detected in the first period, whereas the highest concentrations of PAH8 and PAH16 were found in the third period (P<0.05).

The first lactation period is seen as the period during which more lipophilic contaminants are most concentrated *(Ü, Fig. 1)*.

Finally, the risks of dietary exposure to pollutants detected in milk according to the lactation period in children and adults were evaluated. The calculated mean and maximum EDI, THQ and HI levels are shown in *Table 7*.

Discussion

According to studies conducted in Turkiye, milk yield of these cows is higher than some [27] and lower than some other studies [28]. According to the report of the World

Table 7. Estimated daily intake and potential health risk of target endocrine disruptors via milk

Individuals	Indicator	1. Lactation Period			2. Lactation Period			3. Lactation Period		
		PCBs	*p,p'*-DDE	PAH16	PCBs	*p,p'*-DDE	PAH16	PCBs	*p,p'*-DDE	PAH16
	ADI[a]	**0.01**	**0.5**	**0.5**	**0.01**	**0.5**	**0.5**	**0.01**	**0.5**	**0.5**
Adults	aveEDI[b]	0.0504	0.0069	0.0026	0.04	0.0056	0.0023	0.0454	0.006	0.0029
	aveTHQ[c]	5.04	0.0138	0.0052	4.0	0.0112	0.0046	4.54	0.012	0.0058
	aveHI[d]	5.06			4.02			4.53		
Children	aveEDI	0.181	0.0249	0.0092	0.1435	0.002	0.0083	0.163	0.0214	0.0105
	aveTHQ	18.1	0.0498	0.0184	14.35	0.004	0.0166	16.3	0.0428	0.0210
	aveHI	18.17			14.37			16.364		
Adults	maxEDI[e]	0.318	0.0807	0.0069	0.2489	0.0620	0.0058	0.2843	0.0673	0.00715
	maxTHQ[f]	31.8	0.1632	0.0138	24.89	0.1240	0.0116	28.427	0.1345	0.01430
	maxHI[g]	31.98			25.03			28.58		
Children	maxEDI	1.1420	0.2911	0.0245	0.8929	0,0022	0.0209	1.0206	0.2399	0.0259
	maxTHQ	114.202	0.5822	0.0489	89.292	0.0044	0.0417	102.062	0.4799	0.0518
	maxHI	114.83			89.34			102.59		

***Note:** The risk assessment was done for PCB28, PCB153, and PCB180 among PCBs, and for phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, benzo(b) fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3,c,d)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, and acenaphthene among PAHs.*
[a] Acceptable Daily Intake (µg/kg/day), [b] Average Estimated Daily Intake (µg/kg/day), [c] Average Target Hazard Quotients, [d] Average Hazard Index, [e] Maximum Estimated Daily Intake (µg/kg/day), [f] Maximum Target Hazard Quotients, [g] Maximum Hazard Index

Holstein Federation in 2020, the milk yield of the same breed of cows in Turkiye is lower than in some European countries, Canada, USA and Japan (6.790-12.431 kg). However, it is higher than the milk yield of cows in New Zealand (average 4.492 kg) [29].

While the amount of milk increased, the fat content in the milk decreased. Bedö et al.[30] reported that there is an inverse relationship between milk yield and the fat content in milk, and that as the yield increases, the fat content decreases.

The analytical method recovery levels met the requirements of the SANTE guidelines [17]. According to the SANTE guideline, if the target analytes are at the ppb level, the recovery should be in the range of 50-120% and the RSD values should be less than 15%.

The proportion of the sample exceeding the maximum residue limit (MRL) was 48%, 10%, and 16% in the first, second, and third lactation periods, respectively *(Table 4)*. However, Aslam et al.[8] that total DDTs were detected in 85% of the samples collected from India. This present study shows that the occurrence of EDCs in cow's milk collected in Turkiye is less frequent compared to that in India. In total indicator PCBs (PCB28, PCB138, PCB153, and PCB180), the proportion of the milk sample exceeding the MRLs reported in the European Union (EU) and Turkish Food Codex (TFC) was 10%, 8%, and 12% in the first, second and third lactation periods, respectively. The MRL determined for milk and dairy products in the EU and TFC is 40 µg/kg lipid weight [24,25].

The PAH concentrations detected in the milk indicate the continued presence of PAH sources in the environment. This situation is similar to other studies; it was reported that PAH16 [12] and chrysene [10] were detected in all milk collected from Nigeria and Turkiye. Because PAHs can be found widely in the atmosphere as well as in aquatic and terrestrial systems [31]. PAH4 concentrations were found to be 1.22-0.8, 0.93-0.1 and 0.93-0.1 µg/kg in the first, second and third lactation periods, respectively, which are much higher than the levels found by Kaçmaz in raw milk (0.10-0.06 µg/kg) [10]. However, it is lower than the concentrations detected for PAH16 in free range cow milk collected from Nigeria.

There is no established MRL for PAHs in milk in the EU and TFC. However, the MRL values determined for PAHs, including infant formula and follow-on formula (including infant milk and follow-on milk), are 1 µg/kg lipid weight for benzo(a)piren and PAH4 [benzo(a)piren, benzo(a) anthrasen, benzo(b)fluoranthene and chrysene] [24,25]. When evaluated in terms of these limits, 4% of the milk taken in the first lactation period exceeded the MRLs determined for benzo(a)piren (1.05 and 1.12 µg/kg), while the proportion of the milk sample above the MRLs for PAH4 was 30%, 18%, and 22% for the first, second and third lactation periods, respectively. This may be because the legally established MRL for PAHs is quite low. The proportion of samples exceeding the MRL for PAH4 and benzo(a)pyrene was highest in the first period. This suggests that cows accumulate PAHs during the dry period and gradually eliminate them from their bodies.

There is no MRL in the EU and TFC for DDT. In Codex Alimentarius, the MRL for total DDTs in milk (sum of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD) was determined as 20 µg/kg [26]. Accordingly, the proportion of the sample exceeding the MRL for DDT was determined as 4%, 2%, and 4% for the first, second and third lactation periods, respectively *(Table 5)*.

Normally, the highest concentrations of all target EDCs would be expected during the first lactation period when the fat content is highest. However, the highest PAH concentration was detected in the third lactation period, when the fat content was lower than in the first lactation period. This can be explained by the octanol-water partition coefficient ($logK_{ow}$) of the compounds. As it is known, the higher the $logK_{ow}$ coefficient, the higher the lipophilicity of a substance. Lighter and more hydrophilic PAHs with lower $logK_{ow}$ values (<6) are more water soluble. Among the 16 PAHs, 10 compounds (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthen, pyrene, benzo(a)anthracene, chrysene) have a $logK_{ow}$ value below 6, whereas 6 heavy compounds have a logKow value above 6. The $logK_{ow}$ values for PCB28, PCB138, PCB153, PCB180 and *p,p'*-DDE are 5.69, 7.62, 7.62, 8.27 and 6.0, respectively [32].

The results indicate that the first lactation period is when lipophilic pollutants are most concentrated. This is clearly related to the fat content of the milk. As can be seen, as the fat content in milk increases, the rate of lipophilic EDCs also increases These results are in agreement with the study that found that OCPs detected in human milk colostrum were higher than those collected in other periods [31].

Commercial DDT is a pesticide consisting of a mixture of several isomers and is one of the most important metabolites of *p,p'*-DDE, formed by environmental degradation [33]. In this study, *p,p'*-DDE was found in 28% of the milks in each lactation period. According to the Agency for Toxic Substances and Disease Registry [34], *p,p'*-DDE is the main stable metabolite of DDT. In a study conducted among healthy Chinese mothers, it was reported that the half-life of *p,p'*-DDE in breast milk is 8 months [35]. While the half-life of DDT in soil is 2-15 years, it is more than 150 years in the aquatic habitat [34].

When considered in terms of risk assessment, it is seen that the first lactation period is the riskiest period, followed by the third and second lactation periods. Additionally, it is

seen that the EDI levels calculated for PCBs in the third lactation periods exceed the ADI values determined by the European Food Safety Authority. However, EDI levels remained below the ADI values for PAHs and OCPs. As mentioned before, THQ and HI levels above "1" are indicators of health risk. EDI, THQ, and HI levels are higher than those of adults because children consume more milk than adults and have lower body weight. Thus, in the study conducted with targeted EDCs, it is seen that there is no significant change in the elimination of EDCs in milk during the lactation period, as in aflatoxins, and it is excretion at almost the same rates in each period. Contaminated milk consumption poses a higher risk of children compared to outcomes.

It is seen that endocrine disrupting compounds are excreted more with milk in the first lactation period, followed by the third and second lactation periods. In the evaluation, it was concluded that the elimination was directly proportional to the amount of fat in the milk, and the concentration of endocrine disrupting compounds increased as the amount of fat in the milk increased.

Declarations

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

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Conflict of Interest: The authors declared that there is no conflict of interest.

Ethical Statement: This study does not require ethics committee approval.

Author Contributions: Conceptualization, Funding acquisition, Methodology, Investigation, Formal analysis, Visualization: OK, IS, BYD, UTS, AF; collection of samples, UGB, MT, MAH, REH, AF; performing analysis, OK, UGB, MT, MAH, REH, AF; critical reviews of the manuscript, edition, and provision of important intellectual content and final version approval, all authors.

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RESEARCH ARTICLE

Evaluation of the Characteristics of Congenital Portosystemic Shunts in Dogs and Cats Using Computerised Tomography Angiography and Brain Magnetic Resonance Imaging [1]

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Abstract

Portosystemic shunts (PSS) are abnormal vessels that allow blood to bypass the liver, leading to a variety of clinical symptoms due to the lack of normal hepatic metabolism. Imaging modalities like Computed Tomography Angiography (CTA) and Magnetic Resonance Imaging (MRI) are critical in the diagnosis and characterization of PSS. Nine animals, with clinical presentations suggestive of CPSS, underwent CTA and brain MRI. The CTA was performed using a 32-detector CT unit, and MRI scans were conducted on a 1.5 Tesla Siemens Magnetom Avanto system. Six cases of extrahepatic portosystemic shunts (EPSS) and three intrahepatic shunts (IHPSS) were diagnosed. MRI findings included brain atrophy and white matter hyperintensities, correlating with the type of shunt. The study demonstrates the value of MRI in identifying specific brain changes associated with CPSS. Advanced imaging techniques are indispensable for the accurate diagnosis of CPSS. The study's findings reinforce the need for further research with a larger cohort to establish a stronger correlation between CPSS types and brain MRI changes, aiming to enhance clinical management for affected animals.

Keywords: Cat, Computed tomography, Congenital portosystemic shunt, Dog, Magnetic resonance imaging

Introduction

Portosystemic shunts (PSS) are congenital vascular anomalies that allow the direct passage of portal venous blood into the systemic circulation [1]. There are two main forms: intrahepatic portosystemic shunts (IHPSS) and extrahepatic portosystemic shunts (EHPSS). IHPSS typically originates from the left branch of the portal vein and is associated with the failure of the closure of the ductus venosus. EHPSS, on the other hand originate from a part of portal system which is outside the liver [2-4].

Congenital portosystemic shunts (CPSS) lead to bypass of the ammonia-rich blood away from the liver, and this condition is the most common cause of the hepatic encephalopathy (HE) in cats and dogs [5]. Clinical symptoms of HE include mental alterations, seizure activities, and proprioceptive deficiencies [6]. These symptoms may become more pronounced after the consumption of high-protein foods [7].

It is well known that ammonia is only metabolized in the astrocytes of the brain [8,9]. Astrocytes produce glutamine by the metabolization of ammonia and glutamate by an enzyme called glutamine synthetase which is found in the endoplasmic reticulum of astrocytes [10]. However, in cases of liver failure, increased ammonia levels elevate

the intracellular glutamine level [11,12]. Mitochondrial dysfunction, the formation of "Alzheimer type II astroglia," and changes in cerebral metabolism may occur as a result of this process [8,10].

Computed Tomography Angiography (CTA) has emerged as a more advantageous tool for the diagnosis of PSS in dogs compared to other methods [13]. Advances in Multidetector Computed Tomography (MD-CT) technology have reduced the examination time and the doses of radiation which make computed tomography (CT) more widely used in liver assessments [14]. The progress in MD-CT technology allows for the rapid acquisition of high-resolution dual-phase abdominal CTA which provides detailed information about the abdominal vascular structures [15].

On the other hand, Magnetic Resonance Imaging (MRI) has become an important tool for diagnosing brain pathologies and is increasingly widespread in veterinary medical practice [16]. Existing literature indicates that brain MRI findings are characterized by significant hyperintensity on T1 sequences in the lentiform nuclei of dogs and cats diagnosed with CPSS [11,16,17]. Similar MRI lesions have been observed in humans with chronic HE, and it is thought that these lesions are related to manganese accumulation [17]. Similar findings have also been documented in dogs [17]. Regarding with acute HE, more pronounced and more specific findings can be detected by MRI. In humans, findings such as diffusion restriction, widespread T2/FLAIR hyperintensities in cortex, and T2/FLAIR hyperintensities without any contrast agent accumulation in the thalamic nuclei have been described [18,19].

The aim of this study is to confirm the diagnosis of CPSS in cats and dogs using CTA and to comprehensively compare the clinical complaints of patients with brain MR findings.

Material and Methods

Ethical Statement

This study was approved by the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine Animal Experiments Local Ethics Committee: 2024/02. Consent forms were obtained from all the owners of the patients who came to our clinic on those dates.

Data Collection

The data of our study were used from the images previously obtained from the Istanbul University, Veterinary Faculty, Department of Radiology between 2021 and 2023.

Animals

This study was conducted on animals applied to Animal Hospital of Istanbul University, Faculty of Veterinary Medicine between years 2021- 2023. All animals had the medical history, clinical symptoms, and biochemical values consistent with CPSS. A total of 9 animals, consisting of 5 dogs and 4 cats, were included in this study.

The patients included in the study consisted of cats and dogs of different breeds between the ages of 1-5 years.

Computed Tomography Protocol

Animals which were suspected of having CPSS underwent CTA. Haematological and serum biochemical parameters (Glucose, Creatinine, Urea, Urea/Creatinine, Phosphorus, Calcium, Total Protein, Albumin, Globulin, Alanine aminotransferase, Alkaline Phosphatase, Gamma-glutamyl transferase, Total bilirubin, Cholesterol, Ammonia, Amylase, Lipase) were measured before anaesthesia. Values were found between normal ranges. Furthermore, to confirm the diagnosis and gather more detailed information, brain MRI was performed on the animals. The HE scoring scale was converted from the scoring system previously used by Meyer [20]. In the evaluation of hepatic encephalopathy status, dogs with no clinical signs of hepatic encephalopathy were assigned grade 0; dogs with mild movement disorder, apathy or both were assigned grade 1; dogs with severe apathy, mild ataxia or both were assigned grade 2; dogs with hypersalivation, severe ataxia, head pressing, blindness, rotation or any combination of these signs were assigned grade 3; and dogs with seizures or in stupor or coma were assigned grade 4.

Patients were sedated using 5-7 mg/kg dose of propofol (Propofol-Genthon®) as premedication. Anesthesia was continued with 2% isoflurane (Isoflurane-Adeka®) after intubation.

The scans were conducted using 32-detector CT device (Siemens Somatom Go Now, Germany). All animals were scanned in a dorsal recumbent position. Initially, a topogram image was obtained to determine the scanning area. Subsequently, contrast medium (Opaxol TM) at a dose of 640 mg/kg and a rate of 3 mL/s was administered through a 20-24 gauge catheter via the right or left cephalic vein using a high-pressure injector pump. The threshold contrast enhancement value (100-120 HU) was set by manually marking the aortic lumen to trigger the start of the scan using the "bolus tracking" technique. The scan was automatically initiated when this threshold value was reached.

Arterial phase images were acquired from the caudal thoracic region to the pelvis with a 0.70 mm collimation, 1 mm slice thickness, and a table speed of 1.5 mm per gantry rotation. After the administration of contrast medium, a 30-40 sec delay was allowed, and portal phase images were obtained with the same collimation and slice thickness.

Dorsal, sagittal, maximum intensity projection (MIP), Multiplanar Reformat (MPR), and volume rendering images (VRT) were obtained from the acquired transverse images. Both transverse and reformatted images were evaluated by a radiologist experienced in abdominal radiology.

The MD-CT protocol is presented in detail below:

· Collimation: 16 x 0.75 mm
- Section thickness: 1 mm
- Reconstruction interval: 0.5 mm

· Tube voltage: 120 kV
- Effective tube current: 160 mAs
- Gantry rotation time: 650 msec
- Scan direction: Cranio-caudal
- Scan surface: 12-22cm
- Scan time: 12-18 sec

MRI Examination

All magnetic resonance imaging was performed using a Siemens Magnetom Avanto scanner (Germany) with a magnetic field strength of 1.5 Tesla. For the imaging process, T1-weighted images were acquired with parameter settings of a repetition time (TR) of 350 ms and an echo time (TE) of 12 ms. The field of view for these images was set to 12 x 10 cm, and the matrix size was determined as 200 x 256. The slice thickness was set at 2.5 mm with a slice gap of 0.5 mm.

For the T2-weighted images, the TR and TE values were 3500 ms and 90 ms, respectively. The same field of view was used for these images, but the matrix size was adjusted to 320 x 284.

Enhanced T1-weighted images utilized a gadolinium-based contrast agent gadolinium DPTA (Gadovist-Bayer, Germany) 1.0 mmol/mL. This contrast agent was administered as an intravenous bolus at a dose of 0.1 mmol/kg.

This protocol was employed to ensure the highest image quality and optimal detection of diseases.

Results

Summary of all cases have been shown in *Table 1*. A total of nine cases (5 dogs, 4 cats) were diagnosed with CPSS using CTA in this study. The population of dogs examined in this study consists of entirely small breeds. Seven of these cases, exhibited central nervous system symptoms, while only two cases did not exhibit any of these symptoms. Additionally, approximately 37% of the cases showed gastrointestinal symptoms (vomiting, diarrhea, excessive salivation).

Table 1. *Summary of cases*

Patient	Age	Species/ Breed	Body Weight (kg)	Vena Porta	Vena Hepatica	Shunt Type	Shunt Diameter	Grade for Hepatic Encephalopathy	Fasting NH_3 (µg/ dL)
Case 1	1 year	Dog/ Pekingese	8	Hipoplasic	Normal	Spleno-Caval (EHPSS)	4.8 mm	III	540
Case 2	5 months	Dog/Beagle	10	Hipoplasic	Hipoplasic	Spleno-Caval (EHPSS)	7.1 mm	III	180
Case 3	1 year	Cat/Mixed	3	Hipoplasic	Normal	Spleno-Caval (EHPSS)	4.6 mm	III	244
Case 4	5 years	Dog/Maltese terrier	5	Hipoplasic	Normal	Spleno-Caval (EHPSS)	3.6 mm	III	190
Case 5	7 months	Cat/ Siamesse	1.5	Hipoplasic	Normal	Mesenterico- Caval (EHPSS)	3.9 mm	III	290
Case 6	6 months	Cat/ Siamesse	2	Right portal vein branch is hypoplastic, left portal branch is ectatic	Normal	Between the left portal branch and the left hepatic vein (IHPSS)	5 mm	II	119
Case 7	4 years	Dog/ Yorkshire Terrier	3	Hipoplasic	Hipoplasic	Spleno-Caval (EHPSS)	6 mm	III	378
Case 8	1 year	Dog/ Pomerian	3	Normal	Normal	Between the left portal branch and the left hepatic vein (IHPSS)		0	198
Case 9	1.5 year	Cat/Scottish Fold	3	Hipoplasic	Normal	Porto-Caval Between the left portal vein and the vena cava caudalis (IHPSS)	4.8 mm	IV	325

Reference value: 23-78 µg/dL (for cats), 16-75 µg/dL (for dog)

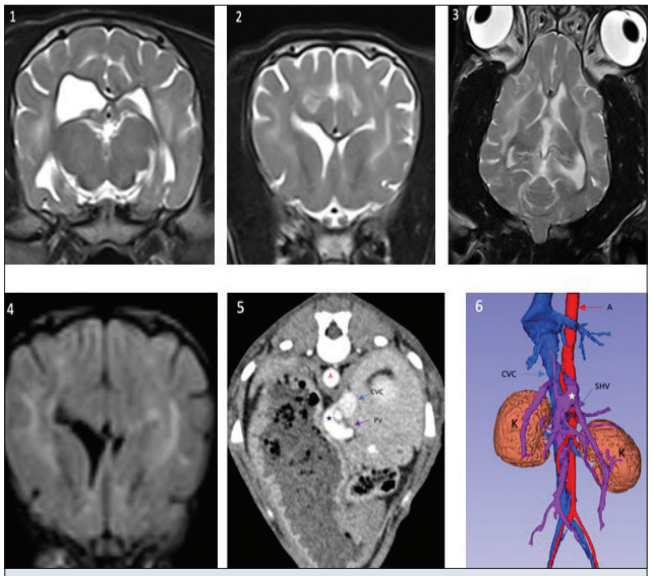

Fig 1. MRI and CT findings of case 2. 1-2: Tranversal and 3: dorsal-T2-weighted MR image showing combined symmetric hyperintensity *(arrows)* in the white matter of the brain, 4: transversal FLAIR MR image showing bilateral and symmetric hyperintensity *(arrows)* affecting the periventricular white matter, 5: transversal CTA showing vascular connection *(asterisk)* between the splenic vein (SHV) and the vena cava caudalis (CVC), (A: Aorta), 6: dorsoventral 3D reconstruction image showing the shunt vessel between CVC and splenic vein

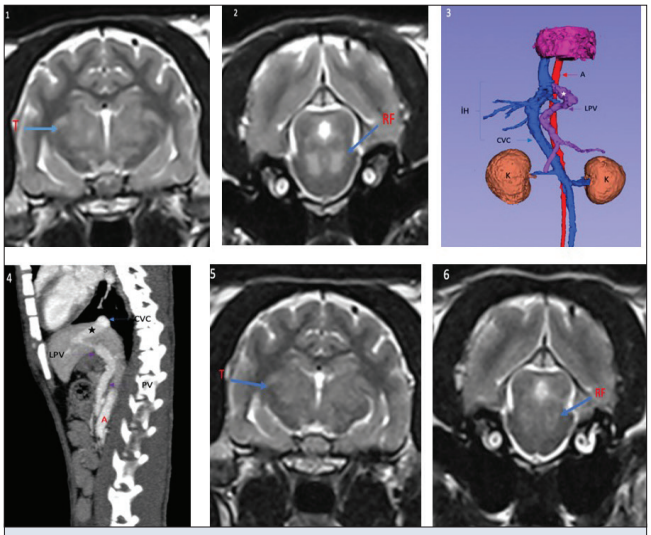

Fig 2. MRI and CT findings of case 9. 1: Transversal T2 W MR image (T) shows hypersensitivity in the thalamus, 2: Transversal T2 W MR image (RF) shows hypersensitivity in the reticular formation, 3: Dorsoventral 3D reconstruction CT image showing the shunt vessel *(asterisk)* between the left branch of the vena porta in the intra hepatic section (IH) and the vena cava caudalis, 4: Lateral CT image showing the shunt connection of the vena porta with the vena cava caudalis *(asterisk)* 5-6: MR imaging after the patient was treated shows that the hypersensitivity in the thalamus and reticular formation decreased (A: Aorta)

The diagnosis of EHPS was observed in six cases (case 1, 2, 3, 4, 5, 7) *(Table 1)*. Five of these cases displayed spleno-caval shunt profiles, while one (case 5) showed a mesenterico-caval shunt profile according to Nelson & Nelson's new morphological decriptions [4]. In case 5 with a mesenterico-caval shunt profile, it was determined that the vena mesenterica caudalis joined the vena cava caudalis (VCC) at the level of L4 vertebra and VCC was dublicated. Five of these cases (case 1, 3, 4, 5, 7) showed atrophic changes such as sulcal and ventricular expansion and a decrease in gyrus volume in the brain. In the brain MRI of case 2, increased signal intensity was observed on T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences of the periventricular white matter regions *(Fig. 1)*. It was determined that all cases diagnosed with EHPS had hypoplastic vena porta.

The diagnosis of IHPS was observed in three cases (case 6, 8, 9) *(Table 1)*. In all of these cases, the integration of left portal vein with VCC was observed. Brain MRI evaluations of these cases revealed the followings: in case 8 (HE grade: 0), no pathological findings were found, while in case 9, significant hyperintense signal changes were detected on T2-weighted, FLAIR, and diffusion-weighted imaging (DWI) sequences in the specific regions of the central nervous system (i.e. pons, mesencephalon, bilateral cerebral peduncles, and bilateral thalamus) *(Fig. 2)*. This patient (case 9) also showed compulsive circling behaviours. Case 6 had a signal increase on T1-weighted image in the lentiform nucleus.

In our study, elevated ammonia concentrations were detected in a significant portion of the participants, surpassing the reference values. With in the scope of this study, there is no evidence of any shunt existence between the portal vein and azygos vein.

Discussion

PSS which causes ammonia-rich blood to bypass away from the liver is one of the main factors contributing to the development HE in dogs and cats. This mechanism led to significant hyperammonemia in some of the animals included in our study.

Hyperammonemia can lead to irreversible damage in the developing brain, such as cortical atrophy, ventricular expansion, demyelination, or hypodensities in gray and white matter. Many studies in humans have reported that acute hepatic or hyperammonemic encephalopathy is considered one of the causes of cortical laminar necrosis, and these effects are reported to be particularly severe in neonatal onset [21-23]. Different pathogenic mechanisms have been shown to be involved, including changes in cerebral energy metabolism [21,24]. In two separate case reports by Spinillo et al.[25] and Moon et al.[26] two dogs had MRI findings of diffuse bilateral symmetric hyperintense signals on T2-weighted in the cerebral cortex. Different images taken at various times in the same studies reported the brain atrophy due to cortical laminar necrosis [20,26]. This corresponds to the brain atrophy symptoms we observed in five out of the nine animals in our study.

However, it is possible to say that this occurred especially as a result of acute hyperammonemia and progressed to chronic effects such as brain atrophy over time. Four of the cases with brain atrophy had spleno-caval shunts in CTA, and one case had a mesenterico-caval shunt (case 5).

Based on CTA findings obtained from the dog (case 2), a diagnosis of EHPS (spleno-caval) was made. On MRI examination, minimal ectasia of the third and lateral ventricles was observed. Remarkably, significant signal intensity increases were observed on T2-weighted and FLAIR sequences in the periventricular white matter of both the left and right hemispheres. However, no pathological contrast fixation was observed after applying intravenous contrast agent.

Human studies have reported that periventricular white matter involvement is observed in acute HE [27-30]. In a more comprehensive study involving 101 human patients, the periventriculer white matter involvement which was devoloped as secondary to acute HE was reported as the most third common finding in MRI [28]. The exact etiology of this condition, in which specific regions of the white matter are affected, is not yet fully understood. Specific areas in white matter regions of the corticospinal pathway or nearby might be more vulnerable to develop secondary edema after liver failure. The underlying reasons for this vulnerability have not yet been definitively understood, but it is thought that the mechanisms such as energy deficiencies or abnormal expression of glutamate transporters might be involved [30].

In our cases which were diagnosed with EHPS, portal vein hypoplasia was observed and were rated as grade III according to Meyer's HE classification [21]. We cosidered that the portal vein volume and PSS volume might have been related to the patient's clinical condition.

Among the brain MRI features of PSS, hyperintensity is prominent on T1-weighted imaging within the lentiform nuclei. These lesions are isointense on T2 and do not show any contrast uptake [11,20]. It is known that these findings regress after PSS repair. Similar lesions have been observed in humans with chronic HE [31].

High manganese concentrations have been found in both blood [32-35] and brain tissues [36,37] of cats and dogs. The literature suggests that manganese accumulation can cause an increase in T1-weighted signal intensity in the lentiform nucleus [15,38]. Although an increase in T1-weighted signal intensity in the lentiform nucleus is the most common finding, it was only observed in one of the cases which were diagnosed as IHPS (case 6) in our study. Since manganese values were not measured in our study, the relationship with our findings could not be evaluated. Among our cases with IHPSs, one dog (case 8; HE grade: 0) did not show any pathological changes in the brain MRI.

Based on the CTA findings of our 1-year-old cat (case 9), a diagnosis of IHPS (between the left portal branch and the VCC) was made. Brain MRI examination revealed significant hyperintense signal intensity changes on T2-weighted, FLAIR, and DWI sequences in the Pons, mesencephalon, cerebral peduncles, and thalamus. These findings were considered to reflect a combination of vasogenic and cytotoxic edema. However, no pathological contrast accumulation was observed. A notable point is that rostral thalamic lesions can lead to compulsive circling behavior in cats [39]. In our study, compulsive circling behaviour was present in cats with thalamic lesions. In addition, a study based on a rat model showed that glucose metabolism was increased maximally in the reticular formation after PSS development, and consequently, caused the development of hyperammonemia [40]. Similar to this, significant hyperintense lesions were observed in the reticular formation in two West Highland White Terrier dogs with PSS [41].

The brain MRI findings of Case 9 such as increased T2A signal in the thalamus and mesencephalon were similar to lesions caused by thiamine deficiency [42]. However, a specific analysis of thiamine levels could not be performed in this current study. The patient's balanced diet and no consumption of supplementary food indicate that thiamine deficiency was not diet-related [43]. A decrease in bile production and consequent digestive and absorption problems may occur as a result of deterioration of liver functions in patients with PSS [44]. Thiamine is stored in liver and play an important role as a co-enzyme in carbohydrate metabolism. Thiamine deficiency can lead to symptoms similar to HE, such as Wernicke's encephalopathy [45]. It should be taken into account that thiamine deficiency may be a potential factor, especially in intestinal malabsorptive states or other chronic enteropathies, which may reduce the absorption of thiamine from the diet.

However, there is no specific evidence of thiamine deficiency or similar brain pathologies in patients with PSS [11,20,28,46]. As a clinical approach in our study, medical treatment and dietary protocol were applied since the patient's owner refused the surgical intervention. B-complex vitamin preparations were added to this treatment. After six months of follow-up, a decrease in the lesions was observed in the patient's brain MRI. In the light of these observations, it is difficult to directly associate these findings with thiamine deficiency or PSS.

In this study, various brain MRI findings were observed in the cases with PSS. Despite methodological similarities, studies on animal models with PSS yield different results and findings. In our study, we also observed findings that reflect the diversity encountered in the literature. However, detailed studies directly comparing shunt type with clinical and MRI findings in animals with PSS are

limited. Within the scope of this study, we examined the potential relationships between the type of PSS and brain MRI findings. With new studies, it will allow a more precise identification of potential lesions in the brain that may be associated with the type of shunt.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (YA) on reasonable request.

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Author Contributions: BD and ZM: Conceived and supervised this study; YK, YA, ZM and MK: Collection of 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

Determination of the Relationship Between Beak Length and Body Weight of Juvenile Northern Bald Ibis *(Geronticus eremita)* and Estimating Body Weight from Beak Length

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Abstract

The aim of the study is to measure the body weight and beak measurements of the juvenile bald ibis birds breeding in Türkiye and try to find out whether there is a comparative relationship between them and compare them with the measurements in the other studies. In this direction, beak measurements and body weights of total 460 bald ibis chicks, including 197 male chicks and 263 female chicks, were determined in an 11-year period from 2012 to 2022. Average upper beak length was found 15.68 cm, lower beak length was 11.34 cm and average body weight was 1.11 kg in male bald ibis juveniles. Average upper beak length was found to be 15.08 cm, the average lower beak length was 10.84 cm and the average body weight was 1.07 kg in female bald ibis juveniles. A positive and moderate significant relationship was found between upper beak length and body weight (P<0.001). It was determined that the average body weight of male bald ibis juveniles was higher than the body weight of female bald ibis juveniles (P<0.001). Body weight variation could be explained by using only upper beak and lower beak measurement in 26% and 29% respectively. No significant variation was observed in the live weight of bald ibis chicks over the years.

Keywords: Bald ibis juveniles, Beak length, Body weight, Estimating weight

Introduction

The northern bald ibis *(Geronticus eremita)* belongs to the Threskiornithidae family of the order Pelecaniformes [1]. According to IUCN 2018 data, there are an estimated 200-249 mature individuals of the northern bald ibis, classified as endangered [2,3]. Historically, nomadic northern bald ibis birds were found in the Middle East and Central Europe during the spring and summer months. During winter, they had a large distribution area extending from the Arabian Peninsula to the African Red Sea coast, reaching Eritrea and Ethiopia [4]. However, their numbers have decreased significantly in recent years due to factors such as destruction of their natural habitats, excessive use of pesticides (especially DDT), and hunting [5,6]. For this reason, conservation and breeding centers have been established to ensure the survival of the northern bald ibis species [7].

There are breeding centers in many different places in the world such as Türkiye, Syria, and Morocco. The bald ibises of Moroccan origin are resident, and those at the Breeding Center in Birecik are semi-wild [8]. They are also known to exist as two wild populations on the Atlantic coast of Morocco and near Tamri [9]. Moroccan and Turkish-origin northern bald ibis birds are genetically different from each other [10]. The northern bald ibis is described as a large, glossy black bird with a length of 70-80 cm (28-31 in), a wingspan of 125-135 cm (49-53 in), and an average weight of 1.0-1.3 kg (35-46 ounces) [11]. In Northeast Africa and Northwest Africa, western individuals of a certain species have an average beak length of 14.1 cm in males and 13.3 cm in females [10,11]. A study has reported that eastern ones in Türkiye of the same species have a shorter beak length, averaging 12.9 cm in males and 12.3 cm in females. A study reported a strong positive correlation between upper beak length and lower beak length in male western cattle herons [12]. Beak length plays an important role in the nutrition of the northern bald ibis. These birds use their beaks to catch creatures such as fish in the water, worms in the soil, and insects, etc. [12]. Birds with longer beaks survived better during the drought period by reaching larger seeds compared to species with shorter

beaks [13]. The length of the beak undergoes modifications according to the diet of the species [14]. Birds fed efficiently and in a balanced way will reach a sufficient body size for migration [15]. In a different study, the body mass of adult males of the northern bald ibis was 1.28 kg, and females were 1.17 kg, while the average weight of both male and female offspring was reported to be 1.02 kg [16]. Body weight plays an important role in migratory birds. During the migration period, birds must reach sufficient body size and complete their energy reserves for successful migration [17]. Young northern bald ibis birds that do not reach maturity before migration have a low chance of survival. Adults are more likely to survive compared to juveniles [18]. Young northern bald ibis birds must reach sufficient body size to be able to migrate together with adults. It is understood that beak length and body weight have an important role in feeding and migration. Based on this information, it will be attempted to determine whether the body weights and beak measurements of juvenile northern bald ibis at the semi-wild breeding station in Türkiye differ from the reported measurements in previous studies. With this study, the body weights and beak lengths of male and female Northern bald ibis chicks will be determined again and evaluated to find out whether there is a relationship between body weights, beak lengths, and genders. It is revealed that the beak lengths and body weights of Northern bald ibis chicks have changed over the last 11 years. With these findings, we also aimed to reveal the unknown features of the species due to the decrease in the number of bald ibises in recent years. This study will increase interest in the protection and breeding of the species and will open the door for further new studies [4,19,20]. With the obtained results, we will determine whether the northern bald ibis juveniles have reached a sufficient size to migrate.

Material and Methods

Ethical Statement

This study was carried out after obtaining ethical approval from the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks, dated 11.01.2023, with approval number E-21264211-288.04-8468166.

Ethical approval was also obtained from the Harran University Animal Experiments Local Ethics Committee Presidency with decision number 2024/001/01 and approval number 296272 dated 15/01/2024.

Study Area and Number of Individuals

The study was performed at the Bald ibis Breeding Center, located in Birecik district of Şanlıurfa Province in southern Türkiye (37°02'N, 37°59'E), situated 3 km north of the Fırat River. In the study, the initial body weights and beak lengths of northern bald ibis juveniles born between 2012 and 2022 were measured when they were taken into cages. Over the course of 11 years, beak measurements and body weights were recorded for a total of 460 northern bald ibis juveniles, 197 males and 263 females. Adult and young northern bald ibis birds are released into the wildlife for breeding in the second week of February each year. They breed in artificial wooden nests mounted on high walls within the Breeding Center and in natural rock nests that were crafted by hand in the same location.

Feeding and Care Program

Each northern bald ibis bird was fed a diet consisting of 100 g of ground beef, 14 g of cheese, 44 g of chick feed, 44 g of carrots, and 0.125 eggs. Additionally, adults and young birds were provided with feed ad-libitum from outside once they began to fly. The main components of their natural diet include insects, spiders, scorpions, worms, snails, fish, amphibians, lizards, and small vertebrates such as snakes, small rodents, and birds [7,21,22]. The chicks hatched after an incubation period of 27-28 days [7]. Male chicks are typically incubated for longer periods than females [23]. Approximately after 45-50 days they can leave the nest and fly [12]. Any northern bald ibis birds that were not sent for migration due to various reasons were returned to the cage in June-July of the same year.

Measurement Process

While all individuals were placed into cages, the gender analysis of the juveniles born in that year was initially conducted at the Biology Department of the Middle East Technical University by collecting their blood in separate 3 mL EDTA tubes from each individual. In recent years, gender analysis has also been conducted at the Harran University Genetic Research Center. After attaching a colored plastic ring to one foot and a metal ring to the other foot of each newborn chick, their body weights were measured using an electronic handheld scale (Portable electronic scale WH-A08) with a sensitivity of 5 g for weights ranging from 1-10 kg. Upper and lower beak measurements were taken using a 50 cm metal L ruler with a precision of 1 cm or 10 mm. Subsequently, the chicks were returned to the main cage. After determining the genders of northern bald ibises chicks, they were recorded. The procedures conducted throughout the working year continued in the same program without any changes.

Statistical Analysis

The relationship between beak lengths and body weights was assessed using the Pearson correlation test. Comparison of beak lengths and body weights according to gender factor was conducted using Independent Samples T Test analysis. Regression analyses were performed to

determine which independent variables best predicted live weight. The best regression equations were obtained using a stepwise procedure. The multiple regression equations were evaluated with the determination of coefficient (R2) and the residual standard deviation (RSD). The relationship between beak lengths across years was assessed using One-way ANOVA and Tukey HSD as post hoc test significant difference (HSD) test as a post hoc analysis, as the data exhibited normal distribution based on the Kolmogorov-Smirnov test. A significance level of P<0.05 was taken into account. All the data were analyzed using the SPSS statistical program (version 28.0).

Results

Average upper beak length was 15.68 cm in male northern bald ibis juveniles and 15.08 cm in female juveniles, while average lower beak length was 11.34 cm in male juveniles and 10.84 cm in female juveniles *(Table 1)*. The average body weight was found to be 1.11 kg in male juveniles and 1.07 kg in female juveniles *(Table 1)*. Significant differences were observed between male northern bald ibis juveniles (M=15.68, SD=1.34) and female juveniles (M=15.08, SD=1.17) in terms of upper beak length. It was found that the average upper beak length of male northern bald ibis juveniles is longer than that of female juveniles (t = 7.294, P<0.01) *(Table 1)*.

There are significant differences between male northern bald ibis juveniles (M=11.34, SD=1.27) and female juveniles (M=10.84, SD=1.01) from standpoint of lower beak length. It has been observed that the average lower beak length of male northern bald ibis juveniles is longer than that of female juveniles (t=5.734, P<0.01) *(Table 1)*. Significant differences were also found in body weight between male northern bald ibis juveniles (M=1.11, SE=0.01) and female juveniles (M=1.07, SE=0.01). It was noted that the average body weight of male northern bald ibis juveniles was higher than that of female juveniles (t=8.245, P<0.01) *(Table 1)*.

It was found that there is a significant, moderate positive relationship between upper beak length and body weight (r=0.542, P<0.001) *(Table 2)*. Similarly, a significant, moderate positive relationship was observed between lower beak length and body weight (r=0.512, P<0.001) *(Table 2)*. As the length of both the upper and lower beaks increases, the body weight also increases. The highest positive correlation was found between the length of the upper beak and the length of the lower beak (r=0.926, P<0.001) *(Table 2)*.

There is a significant difference in the length of the upper beak in male northern bald ibis juveniles across years (F=30.363, P<0.001). In 2012, the average upper beak length differences between male juveniles were significantly longer compared to the years 2016, 2017, 2018, 2019, 2020, and 2021 (2.4 cm, 2.0 cm, 1.6 cm, 2.2 cm, 1.5 cm, 1.5 cm), respectively (P<0.01). The average upper beak length of male juveniles in 2012 was not significantly different from those in 2013, 2014, 2015, and 2022 (P>0.05). In 2014, the average upper beak length differences of male juveniles were significantly different compared to those in 2013, 2016, 2017, 2018, 2019, 2020,

Table 1. *Upper and Lower beak length measurements and body weights measurements*

Measurements	Gender	n	Mean ± SE	Standard Deviation (SD)	Min. - Max.	P-Value
Upper Beak Length (cm)	Male	197	15.68±0.09	1.33	10.00-18.50	<0.01
	Female	263	15.07±0.07	1.16	11.20-18.30	
	Total	460	15.33±0.05	1.27	10.00-18.50	
Lower Beak Length (cm)	Male	197	11.34±0.09	1.27	5.50-14.30	<0.01
	Female	263	10.84±0.06	1.01	8.00-13.70	
	Total	460	11.05±0.05	1.15	5.50-14.30	
Body weight (kg)	Male	197	1.11±0.00	0.10	0.73-1.40	<0.01
	Female	263	1.07±0.00	0.11	0.72-1.43	
	Total	460	1.09±0.00	0.11	0.72-1.43	

Table 2. *Correlation between lower beak length upper beak length and body weight in Geronticus eremita*

Beak Length	Upper Beak Length (cm)	Body Weight (kg)
Lower Beak Length (cm)	.926**	.512**
Upper Beak Length (cm)		.542**

*** P<0.01*

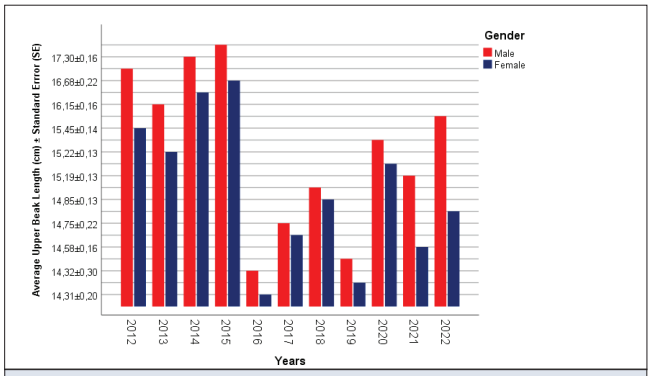

Fig 1. Average upper beak length by years

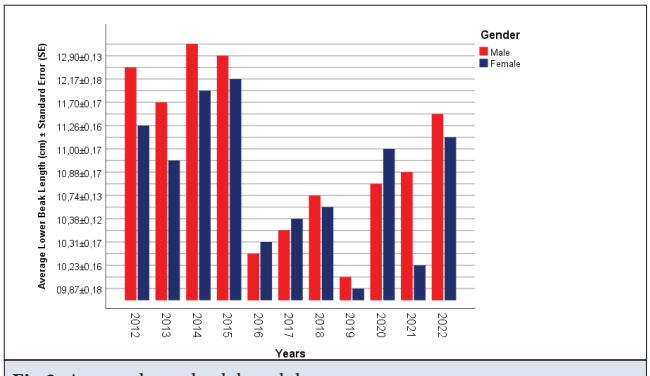

Fig 2. Average lower beak length by years

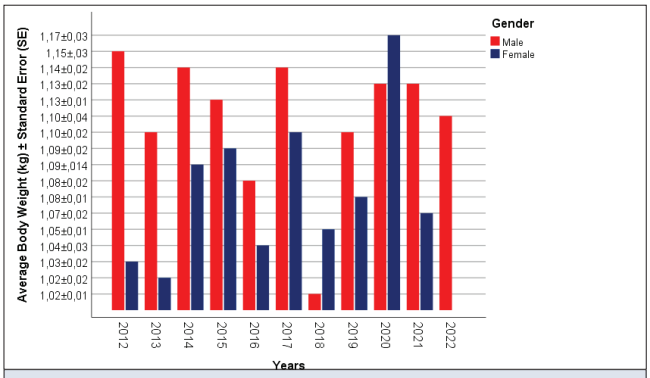

Fig 3. Average body weight by years

2021, and 2022 (P<0.01), with differences of 1.15 cm, 2.9 cm, 2.5 cm, 2.1 cm, 2.7 cm, 2.0 cm, 2.1 cm, 1.8 cm, respectively.

Similarly, there is a significant difference in the upper beak length of female northern bald ibis juveniles across years (F=14.576, P<0.001). In 2012, the average upper beak length differences between female juveniles were significantly longer compared to the years 2016, 2019, 2021, and 2022, which were shorter than 2015 (1.4 cm, 1.3 cm, 0.9 cm, 0.9 cm, 1.2 cm), respectively (P<0.01). The average upper beak length of female juveniles in 2012 was not significantly different from those in 2013, 2014, 2017, 2018, and 2020 (P>0.05) *(Fig. 1)*.

There is a significant difference in the length of the lower beak in male northern bald ibis juveniles across years (F=30.363, P<0.001). The mean lower beak length differences of male juveniles in 2012 were significant compared to those in 2016, 2017, 2018, 2019, 2020, and 2021, respectively (2.0 cm, 1.9 cm, 1.6 cm, 2.2 cm, 1.4 cm, 1.6 cm), (P<0.001). The average lower beak length of male juveniles in 2012 was not significantly different from those in 2013, 2014, 2015, and 2022 years (P>0.05).

Similarly, there is a significant difference in the lower beak length of female northern bald ibis juveniles across years (F=17.735, P<0.001). The mean lower beak length differences of female juveniles in 2015 were significant compared to those in 2012, 2013, 2016, 2017, 2018,

Table 3. *Multiple regression equations for Upper and Lower beaks for predicting live weights of juvenile Geronticus eremita*

Dependent Variable	Gender	Intercept	Independent variables		[a]RSD	[b]R^2	P-Value
			Upper Beak	Lower Beak			
Body Weight (kg)	Male	0.66	0.04	-	0.10	0.21	<0.01
		0.47	-0.003	0.04	0.10	0.24	<0.01
	Female	0.24	0.06	-	0.10	0.32	<0.01
		0.25	0.04	0.02	0.10	0.32	<0.01
	General	0.35	0.05	-	0.10	0.26	<0.01
		0.53	-	0.05	0.10	0.29	<0.01

[a] *RSD: Residual standard deviation,* [b] *R^2: Determination coefficient*

2019, 2020, 2021, and 2022, respectively (P<0.001). The differences were as follows: 0.9 cm, 1.2 cm, 2.1 cm, 1.8 cm, 1.7 cm, 2.4 cm, 1.3 cm, 1.9 cm, 1.4 cm. The average lower beak length of female juveniles in 2015 was not significantly different from that in 2014 (P>0.05) *(Fig. 2)*.

There is no significant difference in body weight among male northern bald ibis juveniles across years (F=1.869, P>0.05). However, a significant difference in body weight was observed among female juveniles (F=4.774, P<0.001). In female juveniles, the body weight difference in 2020 was significant compared to 2013, 2016, 2018, and 2022, being heavier in these years (0.14 kg, 0.13 kg, 0.12 kg, 0.11 kg) (P<0.01). Likewise, the body weight difference in 2017 was significant compared to 2016, 2018, and 2022, with a higher weight in those years (0.09 kg, 0.08 kg, 0.07 kg) (P<0.01). No significant difference was found between the other years (P>0.05) (*Fig. 3*).

Multiple regression equations using upper and lower beak measurements to predict body weight are presented in *(Table 3)*. These equations were developed through multiple linear regression, with beak measurements as the independent variables. It was found that 21% of the variation in body weight was explained by the upper beak alone, and with the addition of the lower beak, the variation increased by 3% in male birds. However, when introducing both upper and lower beak measurements as independent variables in the multiple linear regression equations for female birds, no improvements were observed in predicting body weight. The model including beak measurements explained 32% of the variation in body weight for female birds. Overall, body weight variation could be explained by using upper beak and lower beak measurements alone by about 26% and 29%, respectively.

Discussion

We found that the beak length and body weight of northern bald ibis juveniles reported for Türkiye were different from those reported for Northeast Africa and Northwest Africa. In Western individuals, the average beak length is reported as 14.1 cm in males and 13.3 cm in females. However, in Eastern individuals (in Türkiye), the beak length is shorter, averaging 12.9 cm in males and 12.4 cm in females [10,11]. Our results show that the average upper beak length was 15.68 cm in male northern bald ibis juveniles and 15.08 cm in female juveniles, while the lower beak length was 11.34 cm in male juveniles and 10.84 cm in female juveniles.

It appears that the beak length measurements reported in past studies [10,11] on northern bald ibises in Türkiye are lower than our results (upper beak length: male 15.68 cm, female 15.08 cm; lower beak length: male 11.34 cm, female 10.84 cm). However, since the reported beak lengths are stated as the average beak length, they do not provide detailed results about the exact levels in adult and juvenile individuals. Considering that the beak length of adult individuals is typically longer than that of juveniles, our results suggest that the beak length of the northern bald ibis in Türkiye is not necessarily shorter than that of the northern bald ibis in Northwest Africa. Our results also show a strong positive correlation between lower and upper beak length, which is consistent with the high positive correlation reported by some researchers [24]. Studies conducted on chickens have also reported a positive relationship between beak length and body weight, supporting our findings [25].

When examining the upper and lower beak lengths of northern bald ibis juveniles, we observed an increase from 2012 to 2015, followed by a sharp decrease in 2016. However, from 2016 to 2022, there has been a continuous increase in beak lengths. This pattern is believed to be influenced by environmental factors, such as variations in feeding habits during rainy and drought periods, which can impact beak lengths and body weights. It's important to note that these fluctuations are not hereditary and have varied over the years.

Morphological traits, such as beak size, must be inherited to be considered adaptive [26]. Therefore, the morphological changes observed in the beak length of juvenile northern bald ibis birds are primarily influenced by genetic inheritance from their parents rather than environmental effects.

It was found that the body weight of male northern bald ibis juveniles was higher than that of females. Previous reports revealed that northern bald ibises have an average body weight of 1.0-1.3 kg (35-46 ounces) [11]. The earlier studies on juvenile bald ibises suggested an average body weight of 1.02 kg with no gender difference. However, our findings revealed that the average body weights of male and female juveniles differed, with males being heavier than females [16].

Our findings (1.11 kg for males and 1.07 kg for females) indicated higher body weights compared to those reported by other researchers (1.02 kg) [16]. Previous studies have generally reported an average body weight range of 1.0-1.3 kg (35-46 oz) for bald ibises, and our results align with these findings. However, it's worth noting that these previous studies did not differentiate between adult and juvenile birds [11].

Interestingly, in 2018 and 2020, female bald ibis juveniles were, on average, heavier than male juveniles. This observation suggests that the females may have hatched earlier than the males, a phenomenon observed in other species like chickens, where individuals reaching sexual maturity on time often exhibit higher egg production. This could also be applicable to bald ibises [27].

Based on the results of the multiple regression equation, it can be inferred that upper and lower beak measurements were not strong predictors of body weight in juvenile northern bald ibises, as indicated by determination coefficients ranging from 21% to 32%. These findings suggested that upper and lower beak measurements may not be precise predictors for estimating body weight in juvenile northern bald ibises. Since there is a limited research on using beak measurements to predict body weight in juvenile northern bald ibises, a direct comparison with other studies was not feasible.

We observed that there has been no significant change in the body weight of juvenile bald ibises over the years. However, there have been slight variations in body weights between male and female juveniles, which were not statistically significant. These fluctuations are believed to be influenced by factors such as nutrition and environmental conditions. Providing appropriate dietary supplements to poultry feed has been known to enhance body weight. Despite efforts to protect bald ibises across different regions, their general body weight and beak length have remained stable for an extended period.

From standpoint of nutrition and health management in juvenile birds, the addition of enzymes to their diets has been effective. This approach should be investigated for bald ibises as well [28-31]. However, it's crucial to avoid using additives that could potentially disrupt the intestinal flora [32].

In conclusion, a long beak in bald ibises provides them with a great advantage in capturing their natural food and enhancing their survival abilities. Juvenile bald ibises that are well-fed and have reached sufficient body weight for migration are released to migrate alongside the adults from the breeding station. Their migration success is monitored to ensure that the new generations learn the migration routes alongside the adults.

Declarations

Availability of Data and Materials: The data results obtained in this study are available from the corresponding author and first author upon request.

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Authors' Contributions: AU and AO designed this study. AU: Field work and data collect, AO: Statistical analyze, AU and AO: writing manuscript.

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Research Article

Evaluation of the Effect of Exchange Rate and Energy Prices on Livestock Products and Feed Prices in Türkiye with Path Analysis

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Abstract

The aim of this study was to reveal the effects of exchange rate ($) and energy (oil) prices on livestock product (red meat, milk, eggs) and input (feed) prices in Türkiye through Path Analysis. The material of this study consisted of monthly average exchange rate and energy prices vs. fattening feed, milk feed, and egg feed prices, and producer prices of red meat, milk, and eggs for the years 2010-2023. According to the findings of this study, one unit increase in the exchange rate resulted in a total increase of 0.98, 0.99 and 0.98 units in the prices of fattening, milk and egg feed, respectively; a total increase of 0.93, 0.98 and 0.97 units in red meat, milk and egg prices, respectively ($P<0.05$). It was determined that a one unit increase in energy prices caused a total increase of 0.09, 0.09 and 0.11 units in fattening, milk and egg feed prices, respectively ($P<0.05$). However, energy prices did not affect livestock product prices ($P>0.05$). As a result, it has been observed that the exchange rate affects, particularly, input and livestock product prices, whereas energy prices affect only input costs in Türkiye. Therefore, it is thought that reducing external dependence on energy and upward fluctuations in exchange rates will make significant contributions to the reduction of input costs and product prices in animal husbandry, increase consumption rates and profitability of producers.

Keywords: Livestock products, Energy, Exchange rate, Path analysis, Price

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Introduction

According to data of the United Nations (UN), it is estimated that the world population will reach 8.5 billion in 2030 and approximately 10 billion in 2050 [1,2]. The demand for many basic needs, such as food, clothing, housing, and heating, has increased every day with the increasing population [3]. In addition to increasing the amount of food, it is also necessary to consume food items in an adequate and balanced manner for health purposes. From this perspective, it can be speculated that foods of animal origin are of key importance for adequate and balanced nutrition, especially in underdeveloped and developing countries such as Türkiye. Because animal foods contain substances with high nutritional value such as essential amino acids, valuable oils, vitamins and trace elements, they are vital for a healthy/balanced diet at every stage of life [4].

Food prices are one of the most important criteria for access to animal food [5]. The more affordable and accessible food prices are, the easier it will be for all segments of the society to access food. High production costs, decrease in meadow-pasture areas, global warming/drought, imbalances in the precipitation regime, withdrawals from production (migration), war and natural disasters are among the factors that cause the increase in animal food prices [6]. Increases in production costs depend on the increases mainly in feed, labor, veterinary-health, maintenance-repair, depreciation and other expense items. In addition, the factors that cause the increase in these cost elements include volatility in exchange rates and energy prices in recent years, especially in developing countries such as Türkiye. Continuous and upward changes in energy prices and exchange rates instantly affect the market, and are reflected in an increase in

product prices [7,8]. It is known that these increases cause food inflation and increase general inflation [9].

The Path Analysis method, which was used to determine the effect of exchange rates and energy prices on livestock product and input (fattening feed, milk feed and egg feed) prices in this study, was first introduced by Sewall Wright in the 1930s, and it has been used to determine the statistical relationship between variables in structural equation models. Structural equation models (SEM) are the methods that can be applied in all areas where cause-effect relationships need to be determined. The Path Analysis method, a structural equation model (SEM), is used to determine how much of the effects of independent variables on dependent variables are direct and how much indirect by estimating the structural relationship between numerical variables [10]. In recent years, Path Analyses have been used in many areas [11- 15]. These are marketing and logistics, transportation services, business etc [16-18].

The effect of increasing exchange rates and energy prices on livestock products is a research topic. In this study, it was aimed to reveal the effect of exchange rate and Brent oil prices as energy source on input (fattening feed, milk feed and egg feed) and prices of livestock products (red meat, milk and egg prices) in Türkiye through Path Analysis.

Table 1. *Exchange rate, energy prices, feed prices, red meat, milk and egg producer prices by years (2010-2023)**

Dates	Egg Feed TRY/ kg	Milk Feed TRY/ kg	Fatt, Feed TRY/ kg	Energy (Oil) $/Barrel	Exc Rate $/TRY	Meat Price TRY/ kg	Milk Price TRY/L	Egg Price TRY/ Piece	Dates	Egg Feed TRY/ kg	Milk Feed TRY/ kg	Fatt, Feed TRY/ kg	Energy (Oil) $/Barrel	Exc Rate $/TRY	Meat Price TRY/ kg	Milk Price TRY/L	Egg Price TRY/ Piece
2010-Jan	0.6	0.4	0.4	76.8	1.5	16.8	0.9	0.2	**2017-Jan**	1.2	1.0	0.9	54.6	3.7	25	1.2	0.3
2010-Apr	0.7	0.5	0.5	84.8	1.5	19.2	0.9	0.1	**2017-Apr**	1.2	1.0	0.9	52.3	3.7	26.7	1.2	0.2
2010-Aug	0.7	0.5	0.5	77.0	1.5	18.3	0.9	0.1	**2017-Aug**	1.2	1.0	0.9	51.7	3.5	27.9	1.3	0.2
2010-Dec	0.7	0.6	0.6	91.5	1.5	19.1	0.9	0.1	**2017-Dec**	1.3	1.1	1.0	64.4	3.8	26.1	1.4	0.3
2011-Jan	0.7	0.6	0.6	96.5	1.6	19.1	0.8	0.1	**2018-Jan**	1.3	1.1	1.0	69.0	3.8	26.6	1.4	0.3
2011-Apr	0.8	0.6	0.6	123.3	1.5	18.2	0.8	0.1	**2018-Apr**	1.5	1.2	1.1	72.1	4.1	28.6	1.5	0.3
2011-Aug	0.9	0.7	0.6	110.2	1.7	18.4	0.8	0.2	**2018-Aug**	1.7	1.4	1.3	72.5	5.7	28.9	1.7	0.4
2011-Dec	0.8	0.6	0.6	107.9	1.9	18.8	0.9	0.2	**2018-Dec**	1.6	1.4	1.3	57.4	5.3	27.1	1.7	0.4
2012-Jan	0.8	0.6	0.6	110.7	1.8	18.3	0.9	0.2	**2019-Jan**	1.7	1.4	1.3	59.4	5.4	27.8	1.7	0.4
2012-Apr	0.9	0.7	0.6	119.8	1.8	17.7	0.9	0.2	**2019-Apr**	1.8	1.5	1.4	71.2	5.7	31.0	1.7	0.4
2012-Aug	0.9	0.7	0.7	113.4	1.8	17.4	0.9	0.2	**2019-Aug**	1.8	1.5	1.4	59.0	5.6	31.4	2	0.3
2012-Dec	0.9	0.8	0.7	109.5	1.8	16.7	1	0.2	**2019-Dec**	1.8	1.5	1.4	67.3	5.8	31.4	2.3	0.4
2013-Jan	0.9	0.8	0.8	113.0	1.8	16	0.9	0.2	**2020-Jan**	1.9	1.5	1.4	63.7	5.9	32.8	2.3	0.4
2013-Apr	0.9	0.7	0.7	102.3	1.8	15.9	0.9	0.2	**2020-Apr**	2.2	1.7	1.6	18.4	6.8	36.6	2.3	0.4
2013-Aug	0.9	0.7	0.7	111.3	2.0	15.8	0.9	0.2	**2020-Aug**	2.2	1.7	1.6	44.7	7.3	36.7	2.3	0.4
2013-Dec	0.9	0.7	0.7	110.8	2.1	15.9	1	0.2	**2020-Dec**	2.5	2.2	2.0	50.0	7.7	36.2	2.3	0.7
2014-Jan	1.0	0.7	0.7	108.1	2.2	15.9	1	0.3	**2021-Jan**	2.6	2.2	2.1	54.8	7.4	37.4	2.8	0.6
2014-Apr	1.0	0.8	0.7	107.8	2.1	16.2	1	0.2	**2021-Apr**	2.9	2.5	2.3	64.8	8.2	40.0	2.8	0.6
2014-Aug	1.1	0.8	0.8	101.6	2.2	17.5	1.1	0.2	**2021-Aug**	3.2	2.7	2.5	70.8	8.5	44.5	3.2	0.7
2014-Dec	1.0	0.7	0.7	62.3	2.3	18.4	1.1	0.2	**2021-Dec**	5.5	4.1	3.9	74.2	13.5	61.9	4.7	1
2015-Jan	1.0	0.8	0.7	47.8	2.3	18.9	1.1	0.2	**2022-Jan**	5.4	3.9	3.7	86.5	13.5	65.2	4.7	1V1
2015-Apr	1.1	0.8	0.8	59.5	2.6	20.1	1.2	0.2	**2022-Apr**	7.3	5.6	5.1	104.6	14.7	87.8	5.7	1.3
2015-Aug	1.1	0.8	0.7	46.5	2.8	22.2	1.2	0.2	**2022-Aug**	7.8	6.0	5.6	100.6	18.0	97.4	7.5	1.6
2015-Dec	1.0	0.8	0.7	38.0	2.9	22.6	1.2	0.3	**2022-Dec**	8.0	6.3	5.9	81.0	18.6	114.2	8.5	1.8
2016-Jan	1.0	0.8	0.7	30.7	3.0	24.2	1.2	0.3	**2023-Jan**	8.4	6.7	6.2	82.5	18.8	133.3	10	1.8
2016-Apr	1.0	0.8	0.8	41.6	2.8	24.6	1.1	0.2	**2023-Apr**	8.4	6.8	6.4	84.6	19.3	218.5	10	2.2
2016-Aug	1.1	0.9	0.8	45.8	3.0	25.5	1.2	0.2	**2023-Aug**	9.2	7.6	6.8	86.2	27.0	232.1	11.5	2.3
2016-Dec	1.2	0.9	0.9	53.3	3.5	25.7	1.2	0.4	**2023-Sep**	9.2	7.5	6.7	93.72	26.9	236.5	11.5	2.6

* Dates are spaced

Material and Methods

The study material consisted of monthly average exchange rate ($) and energy (Brent oil) prices, and fattening, milk and egg feed prices as well as red meat, milk and egg producer prices for the years January 2010 - November 2023. The data for exchange rate ($/TRY), energy ($/Barrel), fattening (TRY/kg), milk (TRY/kg) and egg feed (TRY/kg) prices and red meat (TRY/kg), milk (TRY/L) and egg (TRY/piece) producer prices were obtained from the records of official and sectorial institutions such as Central Bank of the Republic of Türkiye [19], U.S. International Energy Agency [20], Turkish Feed Manufacturers Association [21], National Red Meat Council [22], National Milk Council [23] and Egg Producers Central Union [24], respectively (*Table 1*). In this study, the direct effects of exchange rate and energy prices on feed prices (fattening, milk and egg feed) which are considered important inputs [25,26] and red meat, milk and egg prices were examined. In addition, the indirect effects of exchange rates and energy prices on red meat, milk and egg prices through feed prices were also examined. Path Analysis method was used to evaluate the obtained data. The data used in the study were analyzed monthly and are given intermittently in *Table 1*. Model fit values were examined and it was seen that the model fit well for all data [27]. AMOS 24 statistical package program was used in the calculations.

In the Path Analysis method, the direct effect between variables is expressed with standardized regression coefficients (β = Path coefficients). Path coefficient (Pyxk) is found by calculating the effect of the independent variable on the dependent variable [28].

$$Pyx_k = b\frac{Sx_k}{S_y} \quad [1.1]$$

Pxy in the equation expresses the direct effect of the independent variable on the dependent variable.

b: partial regression coefficient;

Sx: Standard deviation of feature x;

Sy: Shows the standard deviation of the y feature.

$$Sx_k = \sqrt{\left[\sum\left(X_{kj} - \bar{X}_k\right)^2\right].\frac{1}{n}} \quad [2.1]$$

$$= \left(\sqrt{\sum X_{kj^2} - \frac{\left(\sum x_{kj}\right)^2}{n}}\right).\frac{1}{n} = \sqrt{S_{xx_k}} \quad [2.2]$$

$$S_y = \sqrt{\sum\left(Y - \bar{Y}\right)^2}.\frac{1}{n} \quad [3.1]$$

$$= \sqrt{\left(\sum Y^2 - \frac{\left(\sum Y\right)^2}{n}\right).\frac{1}{n}} \quad [3.2]$$

$$= \sqrt{S_{yy}} \quad [3.3]$$

Results

In the present study, a Path diagram was initially created, and the obtained model was found to have a good fit. Variance inflation factor (VIF) values were examined for each independent variable, and since the values were less than 10 no multicollinearity problem between the variables was determined. Model fit values for all data were X^2/df: 0.001; normed fit index (NFI): 1.000; comparative fit index (CFI): 1.000; root mean square error of approximation (RMSEA): 0.0001; incremental fit index (IFI): 1.001 and Relative Fit Index (RFI): 1.000. Subsequently, the results were obtained by calculating the Path coefficients *(Fig. 1)*.

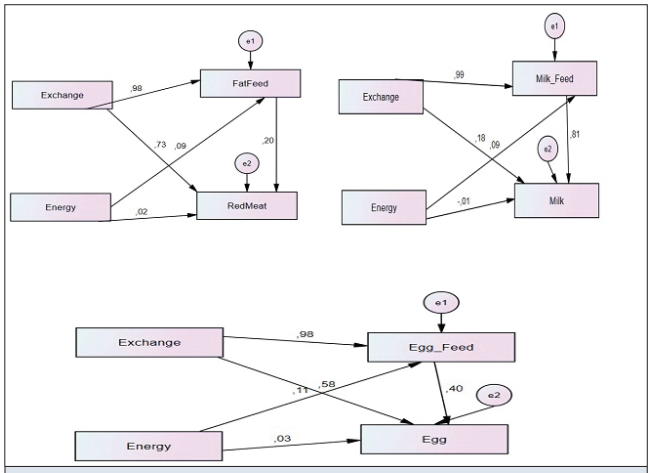

Fig 1. LPath diagram for the dependent variables red meat, milk, egg, fattening feed, milk feed, egg feed prices and independent variables energy prices (oil) and exchange rate

In the present study, Path coefficients and results of analyses showing the relationship between independent variables (exchange rate and Brent oil prices) and dependent variables (fattening feed prices, red meat prices) were presented in *Table 2* and *Table 3*.

Table 2. *Results of Path coefficients of independent variables and red meat and fattening feed prices*

Structural Relationships			β	S_x	C.R.	R^2	P
Fatt. Feed Price	←	Exchange Rate	0.984	0.004	81.97	0.98	0.001
Fatt. Feed Price	←	Energy Price	0.093	0.001	7.767		0.001
Red Meat Price	←	Energy Price	0.023	0.058	0.708	0.87	0.479*
Red Meat Price	←	Exchange Rate	0.734	1.444	4.024		0.001

β: Standardised Regression Coefficients; Sx: Standard Error; C.R: Critical Ratio; * P>0.05

In this study, in which the direct effects were evaluated, according to the results of the structural equation model, there was a positive relationship between exchange rate (β=0.984) and energy prices (β=0.093) and fattening feed prices. The 98% of the total change in fattening feed

Table 3. *Results of direct, indirect and total effects of independent variables on red meat and fattening feed prices*

Standardised Direct Effects			Standardised Indirect Effects		Standardised Total Effects	
Prices	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**
Fatt. Feed	0.984	0.093	0.000	0.000	0.984	0.093
Red Meat	0.734	0.023	0.197*	0.019*	0.931	0.042

* P>0.05

price was explained with the exchange rate and energy prices (R^2=0.98). In the second structural model, it was determined that the 87% of the total change in red meat was explained by the exchange rate (R^2=0.87) and the relationship between the two variables was positive (P=0.001; *Table 2*).

When the exchange rate used in this study, the standardized values of the direct effects of Brent oil prices on fattening feed prices and red meat prices and the indirect effects of exchange rate and energy prices on red meat prices through fattening feed prices were examined, it was observed that the exchange rate had a direct effect on fattening feed prices (β=0.984). It was determined that the direct and indirect effect of the exchange rate on red meat prices was 0.734 and 0.197 units, respectively. It was observed that energy prices did not have a significant effect on red meat prices (P>0.05), whereas it had a direct effect of 0.093 units on fattening feed prices. One unit increase in the exchange rate led to a total (direct + indirect effect) increase of 0.98 unit in fattening feed and a total (direct + indirect effect) increase of 0.93 unit in red meat. It was determined that one unit increase in energy prices caused a total (direct + indirect effect) increase of 0.09 unit in fattening feed *(Table 3)*.

Path coefficients and analysis results showing the relationship between independent variables (exchange rate, energy prices) and dependent variables (milk feed prices, milk prices) were presented in *Table 4* and *Table 5*.

Table 4. *Results of Path coefficients of independent variables and milk and milk feed prices*

Structural Relationships			β	S_x	C.R.	R^2	p
Milk Feed Price	←	Exchange Rate	0.985	0.004	87.872	0.98	0.001
Milk Feed Price	←	Energy Price	0.091	0.001	8.151		0.001
Milk Price	←	Exchange Rate	0.182	0.039	2.07	0.97	0.038
Milk Price	←	Energy Price	-0.006	0.001	-0.42		0.675*

β: Standardised Regression Coefficients; Sx: Standard Error; C.R: Critical Ratio; * P>0.05

A positive relationship was determined between the exchange rate (β=0.985) and energy prices (β=0.091) and milk feed prices. The exchange rate and energy prices explained 98% of the total change in milk feed prices (R^2=0.98). The 97% of the change in milk prices was explained by the exchange rate, and a positive relationship was found between milk prices and exchange rate (β=0.182; R^2=0.97; P<0.05; *Table 4*).

Table 5. *Results of direct, indirect and total effects between independent variables, milk and milk feed prices*

Standardised Direct Effects			Standardised Indirect Effects		Standardised Total Effects	
Prices	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**
Milk Feed	0.985	0.091	0.000	0.000	0.985	0.091
Milk	0.182	-0.006	0.796*	0.074*	0.978	0.068

* P<0.05

When the standardized values of the direct effects of exchange rate and energy prices on milk feed prices and milk prices, and the indirect effects of exchange rate and energy prices on milk prices through milk feed prices were examined; it was seen that the exchange rate's direct impact (prediction) power on milk feed and milk prices was 0.985 and 0.182 units, respectively. It was determined that the indirect impact (prediction) power of the exchange rate on milk prices was 0.796 units. The direct impact (prediction) power of energy prices on milk feed prices was 0.091 units, and it did not directly affect milk prices (P>0.05). However, it was determined that it indirectly affected milk prices through milk feed prices (β=0.074; P<0.05). It was observed that one unit increase in the exchange rate caused a total (direct + indirect effect) increase of 0.99 and 0.98 units in milk feed prices and milk prices, respectively. One unit increase in energy prices caused a total (direct + indirect effect) increase of 0.09 unit in milk feed prices *(Table 5)*.

The relationship of the variables (exchange rate, energy, egg feed, egg prices) with each other, Path coefficients and analysis results were given in *Table 6* and *Table 7*.

According to the findings obtained from the structural equation model, a positive relationship was determined between exchange rate (β=0.978) and energy (β=0.108)

Table 6. *Results of Path coefficients of independent variables and egg and egg feed prices*

Structural Relationships			β	S_x	C.R.	R^2	p
Egg Feed Price	←	Exchange Rate	0.978	0.006	71.173	0.97	0.001
Egg Feed Price	←	Energy Price	0.108	0.001	7.856		0.001
Egg Price	←	Exchange Rate	0.582	0.008	6.513	0.96	0.001
Egg Price	←	Energy Price	0.033	0.000	1.781		0.075*

β: Standardised Regression Coefficients; Sx: Standard Error; C.R: Critical Ratio; * P>0.05

prices and egg feed prices. It was observed that the 97% of the total change in egg feed price was caused by exchange rate and energy prices (R^2=0.97).

There was a positive relationship between egg prices and exchange rate (β=0.582). The 96% of the total change in egg prices was explained by the exchange rate (R^2=0.96; P=0.001; *Table 6*).

Table 7. *Results of direct, indirect and total effects of independent variables on egg and egg feed prices*

	Standardised Direct Effects		Standardised Indirect Effects		Standardised Total Effects	
Prices	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**	**Exc. Rate**	**Energy (Oil)**
Egg Feed	0.978	0.108	0.000	0.000	0.978	0.108
Egg	0.582	0.033	0.391*	0.043*	0.974	0.076

* P<0.05

When the standardized values of the direct effects of the independent variables (exchange rate, energy prices) on the dependent variable (egg feed prices and egg prices) and the indirect effects of these independent variables on egg prices through egg feed prices were examined, the direct impact (prediction) power of exchange rate on egg feed and egg prices was determined as 0.978 and 0.582 units, respectively. It was found that the exchange rate's indirect influence (prediction) power on egg prices was 0.391 units. The direct impact (prediction) power of energy prices on egg feed prices was determined to be 0.108 units, whereas energy prices did not directly affect egg prices (P>0.05). However, it was determined that energy prices indirectly affected egg prices through egg feed prices (P<0.05). It was determined that one unit increase in the exchange rate led to a total (direct + indirect effect) increase of 0.98 unit in egg feed prices and a total (direct + indirect effect) increase of 0.97 unit in egg prices. One unit increase in energy prices caused a total (direct + indirect effect) increase of 0.11 unit in egg feed prices *(Table 7)*.

Discussion

Access to food has become even more difficult as there have been serious problems in the production and supply chain owing to the crises experienced in recent years (pandemics, earthquakes, droughts, global wars, etc.). Problems in the production and supply chains have caused an increase in food prices, which has made it difficult for people to access food. In addition to these problems, increases in exchange rates and energy prices, especially in developing countries such as Türkiye, have caused an increase in the costs of food production and supply and an increase in food prices. This situation has made it even more difficult for people, in Türkiye as in almost all countries around the world, to access food [29].

In the present study, it was observed that the exchange rate had an effect on red meat prices, whereas energy prices did not have a significant effect on red meat prices. The main reasons why the increases in the exchange rate affect the prices of red meat are that live animal and red meat imports are carried out with the exchange rate, and the cost elements in beef cattle breeding are affected by changes in the exchange rate and the depreciation of the TRY against the US dollar every day. It is thought that the lack of the effect of changes in energy prices on red meat prices due to a small share of energy in production costs in beef cattle enterprises where red meat is produced. Both the fact that the biggest cost element in beef cattle breeding is not feed but fattening material [30-32] and the energy cost of enterprises in the feed plant production phase are proportionally limited compared to fertilizer and drug costs. This may have prevented the increases in energy prices from being reflected in red meat prices. On the other hand, it suggests that the unstable environment in the supply of red meat due to imports, problems in dairy cattle farming (milk market is not regulated, diseases etc.), production costs and subsidies may be one of the factors affecting the market prices of red meat, in addition to exchange rates and energy prices [33,34]. In this study, it was determined that the exchange rate had a direct effect on milk and egg prices. It was seen that energy prices indirectly affect milk and egg prices through feed prices. It is known that the biggest expense item in dairy cattle and egg poultry farming is feed cost [35-37]. Therefore, it is seen that the increases in feed prices in dairy cattle breeding and egg poultry farming are reflected in the prices of products obtained from these sectors. In addition, it is thought that changes in feed prices affect milk prices because of lesser pasture use in dairy cattle farming compared to beef cattle farming. The majority of inputs in dairy cattle and egg poultry farming have been affected by the exchange rate, which has increased production costs, and product prices have increased as a result of this cost increase. Changes in energy prices may have affected livestock product (milk, egg) prices by affecting transportation costs (feed supply and production stages) [38]. In addition, countries dependent on foreign oil for Brent oil suffer from both changes in energy prices and negative effects on the exchange rate since they import oil in foreign currencies. Supporting the findings of the present study, several studies have indicated that increases in oil prices indirectly increase food prices [39-42].

This study will help both producers and policy makers to estimate how much input costs and livestock product prices in livestock production may be in the future by looking at the data of institutions Central Bank, etc.) that makes future exchange rate predictions.

In conclusion, Türkiye is a developing country with foreign dependence on energy and high exchange rate

fluctuations. Therefore, it was observed that changes in exchange rates and energy prices affected input and livestock product prices. Fluctuations in exchange rates, foreign dependency and depreciation of TRY can be reduced with the right policies, consumption can be increased by preventing the increase in food prices, and the profitability of producers can be ensured by reducing costs.

Declarations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (E. Aydın).

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Research Article

Evaluation of Efficiency of Thyme Oil, *Cinnamomum verum, Melaleuca viridiflora, Syzygium aromaticum* Essential Oils, and Amitraz for *Varroa* Mite (Acari: Varroidae) Control in Honey Bee (Hymenoptera: Apidae) Colonies Under Field Conditions

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Abstract

Varroosis is a disease that can be observed in all life stages of honey bees and causes serious clinical findings in infested hives. This study aimed to investigate and compare the efficiency of thyme oil, *Cinnamomum verum*, *Melaleuca viridiflora, Syzygium aromaticum* essential oils, and amitraz against Varroosis in the field. After the essential oils were dissolved in glycerin, they were impregnated on strips and applied by placing them between frames. Amitraz was applied by fumigation. Positive and phoretic *Varroa* negative control groups were also included. Treatments were applied once a week for four weeks during the autumn season. The rates of acaricide efficacy and weekly mite count per bottom frame were determined for each of the treatments. According to the results, the highest effectiveness against Varroosis was detected in the amitraz treatment group at 81.3%. The *C. verum* treatment group had the highest efficacy of all the essential oil treatment groups at 73.5%. The efficacies in thyme oil, *M. viridiflora*, and *S. aromaticum* essential oils treatment groups were 71.9%, 71.3%, and 67.4%, respectively. According to the findings of the study, natural extracts can be used instead of chemical drugs in the fight against Varroosis. No toxicity or unnatural death was observed in honey bees throughout the study.

Keywords: Honey bee, Varroosis, Thyme oil, *Cinnamomum verum*, *Melaleuca viridiflora*, *Syzygium aromaticum, Varroa destructor*

Introduction

Honey bees serve a significant ecological function by pollinating the majority of closed-seeded plants [1]. This pollen-emitting insect is also highly desired since its cultivation provides crops with significant nutraceutical benefits [2]. Bee colonies are disappearing all across the world for a variety of reasons [3]. Although there are various reasons for these losses, the principal ones are caused by the *Varroa* agent and the diseases that it causes [4].

Varroa destructor, a parasitic mite, causes significant economic losses by damaging honey bee colonies [5]. The life cycle consists of 4 stages: egg, protonymph, deutonymph and adult [6]. *V. destructor* agents primarily feed on fat body, they also feed on a small amount of hemolymph. *V. destructor* causes weight loss, shortening of lifespan, learning, memory, behavioral disorders, carrying viruses, and immunosuppression in infected individuals [7]. If untreated, colonies of honey bees often do not survive for longer than two years [8]. Colonies infected with *V. destructor* and untreated colonies have lower yield characteristics, especially honey, compared to treated colonies [9]. Acaricides developed from past to present for the control of *V. destructor* are divided into two groups: hard and soft acaricides. The first group includes synthetic drugs such as taufluvalinate, flumethrin (pyrethroid), coumaphos (organophosphate), and amitraz (formamidine). Organic compounds, such as oxalic acid, formic acid, and essential oils, are most typically utilized in the second group [10]. Although traditional pest management options based on synthetic pesticides are popular due to their ease of administration

and superior capacity to decrease insect impact, excessive use endangers honey bee health. Some acaricides can accumulate in wax due to their lipophilic properties. Since hive products can retain residual chemicals, adult and juvenile honey bees can be exposed to these chemicals over long periods of time [11]. Even very low doses or concentrations of these chemicals have been shown to cause non-lethal changes in the neurological, metabolic, physiological and/or behavioural characteristics of honey bees [12]. The colony could face non-fatal effects. The hive may gradually become less populous. Furthermore, the resistance phenomenon has made these acaricides less effective [13].

Efforts are being made to eliminate the residue problems caused by pesticides, the resistance problems that develop against them and to reveal environmentally friendly compounds. As a result, essential oils and their monoterpenes are being extensively researched as potential pest management alternatives. In comparison to hard acaricides, essential oils (EOs) were carefully tested and proven to be long-term effective as miticides against *V. destructor* [14].

Essential oils generally work maximum against *Varroa* agents at temperatures between 20-25°C. However, in honey bee farming, the use of essential oils is ignored at low temperatures, especially in October and November before the winter season, and products such as oxalic acid are used. With our study, we tried to reveal whether essential oils can be used at low temperatures. The current study evaluated alternative techniques for controlling Varroosis by examining the acaridial activity of essential oils (Thyme oil, *Cinnamomum verum*, *Melaleuca viridiflora,* and *Syzygium aromaticum* essential oils) and amitraz in the field.

Material and Methods

Ethical Statement

This study does not require ethics committee approval.

Study Area

The study was conducted in October 2023 at the Balikesir Queen Bee and Bee Products Production Center. The study took place in an apiary with 950 hives. Balikesir is located in the Marmara Region of Türkiye.

Honey Bee Information

Studies were carried out on the *Apis mellifera anatoliaca* breed. The queen bees in each hive were one year old. The honey bee colonies used in the research had a population of approximately 15.000 to 20.000 individuals. Five-frame hives were included in the study. There are 2-3 brood frames and 1-2 frames with eggs in each of them (although no eggs are observed in all of them, there are at least a handful of eggs in each). It was stated that no chemicals or plant extracts were used in the treatment of diseases and pests in the colonies used in the research.

Field Experiment

The powdered sugar method was used to determine whether 150 hives were positive or negative for Varroosis [15]. In order to be placed in the Varroa test apparatus, 300-350 worker honey bees were taken from the combs, brushed and took in the 900 ml apparatus. Fifteen g of powdered sugar was filtered through a sieve. The jars were rolled to distribute the sugar evenly among the bees. After one minute, the jar and honey bees were shaken rapidly on a white paper plate for approximately four minutes, and the *Varroa* displaced during this time were counted [16]. To show field work Varroa tester apparatus and *Varroa* macroscopic appearance are included in *(Fig. 1, Fig. 2)*, respectively. Before each trial, the white paper placed in the pollen traps was renewed in order to count the *Varroa* mites.

General Information About Essential Oils and Amitraz

The essential oils used in the study were obtained from NU-KA DEFNE Essencia company. The essential

Fig 1. *Varroa* tester apparatus

Fig 2. *Varroa* macroscopic appearance *(red arrow)*

oils used were obtained through steam distillation. The information about thyme oil; 100% purity, active ingredients: thymol (48.50%), γ-terpinene (31.35%), and p-cymene (8.25%), linear formula: 2-[$(CH_3)2CH]C_6H_3$-5-$(CH_3)OH$, molecular weight: 150.20 g/mol. *C. verum* oil; 100% purity, active ingredients: linalool (36.0%), methyl eugenol (12.8%), limonene (8.3%), α-terpineol (7.8%) and terpinen-4-ol (6.4%), linear formula: $C_{11}H_{12}O_2$, molecular weight: 132.16 g/mol. *M. viridiflora* oil; 100% purity, active ingredients: 1,8-cineole (31.5%), viridiflorol (21.7%), α-pinene (17.9%), α-terpineol (6.5%), terpinene-4-ol (2.6%), γ-terpinene (2.3%), β-pinene (1.9%), and ledol (1.3%), linear formula: $C_{10}H_{16}$, molecular weight: 136.234 g/mol. *S. aromaticum* oil: eugenol (76.8%), beta-caryophyllene (17.4%), alpha-humulene (2.1%), and eugenyl acetate (1.2%), linear formula: $C_7H_{12}ClN_3O_2$, molecular weight: 205.642 g/mol. Vamitrat VA® is a commercial product containing amitraz (N-methyl-bis(2,4-xylyliminomethyl). Each strip contains 20 mg amitraz.

Preparation of Essential Oils

A total of 180 mL of solution was prepared by mixing 45 mL of essential oil with 135 mL of glycerin. Forty-two strips measuring 2x10 cm were placed inside and waited for five minutes [17]. All essential oils applied in the study were prepared according to the following method. Each strip contains 1.07 mL of essential oil and 3.21 mL of glycerin solution. A total of 84 strips were prepared for each essential oil.

Amitraz Treatment Group

The amitraz treatment group consisted of 7 Langstroth-type hives (each hive consists of 5-6 frames), as in the essential oils treatment and control groups. Amitraz application is shown in *Fig. 3*. After the amitraz strip was placed in the bee smoker, it was burned and applied to each hive through the hive entrance hole with 10 bee smoker movements. Amitraz was used four times in total, on days 0, 7, 14 and 21. Amitraz was applied close to the evening hours, when the bees returned to the hive.

Essential Oils Treatment and Control Groups

Fourty-two honey bee colonies in Langstroth type hives with five or six frames each were divided into six homogeneous groups, four essential oil treatment groups, one phoretic *Varroa* negative control group and one positive control group. There were 7 hives in each group. The essential oils treatments were administered weekly during for four weeks in autumn. Essential oil application was performed on days 0, 7, 14 and 21. Three strips of essential oil were applied to each hive and the strips were

Fig 3. Amitraz application

renewed every week. Count the number of mites in the powdered sugar were applied on days 0 (before treatment) and 28 (post-treatment) to determine *Varroa* loads in honey bee colonies. Similar to the treatment groups, count the number of mites in the powdered sugar were performed on days 0 and 28 in both the negative and the positive control groups, and *Varroa* agents that fell into the pollen trap were counted on days 7, 14, 21, and 28 [18]. Essential oils were applied close to the evening hours, as was the case with amitraz.

While creating the groups, attention was given to distribute the phoretic *Varroa* loads numerically homogeneous in all 7 hives on day 0.

Determination of Treatment Efficacy

The Henderson-Tilton formula was applied to evaluate the therapeutic efficacy of the essential oils and amitraz [18].

$$\text{Corrected \%} = \left(1 - \frac{\text{n in Co before treatment x n in T after treatment}}{\text{n in Co after treatment x n in T before treatment}}\right) \times 100$$

(where n=mite population, T=treated, Co=control).

Statistical Analysis

The statistical analyses were performed utilizing the IBM SPSS20 software. To determine the effects of the agents used in this study on *Varroa*, the One-Way ANOVA test analyzed the Henderson-Tilton treatment efficacy. Comparisons between agents were made with the Tukey-HSD post-hoc test. *Varroa* agents that fall into the pollen trap on different days and the interactions between the factors were evaluated with the Repeated Measures ANOVA test. Determination of statistical significance and whether the test assumptions were met were evaluated according to Mauchly's test results. Since the result of Mauchly's test was less than 0.05 and the epsilon value was less than 0.75, Greenhouse-Geisser correction was applied. Pairwise comparisons between days were determined by applying Bonferroni correction. In comparisons between groups, the Tukey multiple comparison test was used since the homogeneity of variances test assumptions were met. Differences between groups were evaluated as $P<0.05$ and data were presented as mean and standard error [19].

Results

As a result of field studies of 150 honey bee colonies using powdered sugar method, 108 positive (72%) and 42 negative (28%) colonies were detected in terms of *Varroa*. Beyond a threshold of three phoretic *Varroa* mites per 100 bees, the decrease in performance is correlated with the *Varroa* load. Colonies with a load of ten *Varroa* or more on live bees were treated [20]. Hives below ten *Varroa* load were not included in the positive control and treatment groups. Of the 42 *Varroa* positive hives, 28 were included in the four essential oil treatment groups, seven in the amitraz treatment group and the remaining seven in the positive control group. Seven out of the 108 *Varroa*-negative hives (phoretic *Varroa* was not detected these hives) were selected as the negative group in the study, considering their clinical status (clinically healthy hives where *Varroa* is not seen in adult bees, where there are no paralyzed bees or bee deaths in front of the hive). Of mites number of *Varroa* in the powdered sugar shake were applied on day 0 (before treatment) and day 28 (post-treatment) to determine the *Varroa* loads in colonies of honey bees, and the results are given in *Table 1*. On days 0

Table 1. *Day 0 (before treatment) and Day 28 (after treatment) powder sugar count results and treatment efficacies*

Hive No	Amitraz		Thyme oil		*C. verum*		*M. viridiflora*		*S. aromaticum*		Positive Control Group		Negative Control Group	
	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.
1	40	11	43	15	40	17	44	20	46	40	45	58	0	13
2	40	15	34	21	33	16	36	18	38	18	32	40	0	23
3	45	10	48	18	45	20	51	20	53	20	44	48	0	26
4	20	8	21	10	19	10	23	10	25	20	17	36	0	25
5	30	10	19	10	17	9	20	10	22	12	16	48	0	21
6	50	15	58	20	56	15	60	23	62	17	55	69	0	22
7	50	6	51	18	49	13	53	19	53	15	45	70	0	19
Total	**275**	**75**	**274**	**112**	**259**	**100**	**287**	**120**	**299**	**142**	**254**	**369**	**0**	**149**
Days	*Varroa* percentage reductions in treatment groups compared to days 0 and 28													
0-28	Amitraz VCR: 72.72			Thyme oil VCR: 59.12			*C. verum* VCR: 61.38			*M. viridiflora*: 58.18			*S. aromaticum*: 52.50	

D.0: Day 0, D.28: Day 28, V.L:. Varroa Load, VCR: Varroa Count Reduction

Table 2. *Treatment groups pollen trap count results*

Hive No	Amitraz				Thyme oil				*C. verum*				*M. viridiflora*				*S. aromaticum*			
	D.7	D.14	D.21	D.28	D.7	D.14	D.21	D.28	D.7	D.14	D.21	D.28	D.7	D.14	D.21	D.28	D.7	D.14	D.21	D.28
1	40	38	35	32	25	20	18	17	23	18	15	14	27	22	20	18	30	28	25	23
2	53	50	45	40	30	25	20	19	27	22	18	16	36	30	25	23	40	35	30	28
3	68	65	55	50	39	35	30	27	35	30	25	23	45	40	35	33	50	45	40	38
4	30	28	25	22	13	10	9	8	12	9	8	7	15	13	12	10	18	15	13	12
5	28	25	23	20	17	15	13	11	15	12	11	10	18	15	14	12	20	17	16	15
6	65	60	55	50	45	41	40	35	40	37	30	25	50	43	40	34	60	55	50	45
7	55	49	45	40	38	32	30	23	35	30	26	21	40	34	30	25	50	43	40	35
Total	**339**	**315**	**283**	**254**	**207**	**178**	**160**	**140**	**187**	**158**	**133**	**116**	**231**	**197**	**176**	**155**	**268**	**238**	**214**	**196**

and 28, the reduction in *Varroa* numbers was compared. According to *Table 1*, the greatest reduction in phoretic *Varroa* load on days 0-28 was calculated as the amitraz treatment group (72.72%), and the least reduction was calculated as the *S. aromaticum* oil treatment group (52.50%). The number of phoretic *Varroa* increased in both the positive control group and the phoretic *Varroa* negative control group from the beginning to the end of the field studies *(Table 1)*. Additionally, all hives in the phoretic *Varroa* negative control group were *Varroa*-positive two weeks after the beginning of the study. The treatment efficacies of amitraz, thyme oil, *C. verum*, *M. viridiflora*, and *S. aromaticum* oils were calculated using the Henderson-Tilton formula to the counts on days 0 and 28, and the results were 81.3%, 71.9%, 73.5%, 71.3%, and 67.4%, respectively. Among the treatment groups, the highest number of *Varroa* falling into the pollen trap was detected in the amitraz treatment group, while the lowest number was detected in the *C. verum* treatment group. In all treatment groups, the number of *Varroa* falling into the pollen trap decreased from the day 7 to the day 28 *(Table 2)*. In addition, in the positive control group, *Varroa* agents were detected in the pollen traps on all counting days, while in the phoretic *Varroa* negative control group, *Varroa* agents were not observed in the pollen traps on day 7 and day 14, while *Varroa* agents were detected on day 21 and day 28 *(Table 3)*. According to Henderson-Tilton efficacy findings for the agents amitraz group has the highest treatment effectiveness (82.32±2.24), while the lowest treatment effectiveness (66.37±6.54) was calculated as the *S. aromaticum* treatment group. Also, treatment effectiveness were found as (73.65±3.99) in *M. viridiflora* treatment group, (72.42±3.72) in *C. verum* treatment group, (71.34±3.98) in thyme oil treatment group, respectively. The highest number of phoretic *Varroa* in pollen traps was detected in the amitraz group (1st count=48.42±6.08, 2nd count=45.00±5.79, 3rd count = 40.42±4.98, 4th count=36.28±4.61) while the least was observed in the *C. verum* (1st count = 26.71±4.03, 2nd count = 22.57±3.89, 3rd count = 19.00±3.12, 4th count = 16.57±2.55) treatment group. Also, number of phoretic *Varroa* in pollen traps were found in the *S. aromaticum* treatment group (1st count = 38.28±6.11, 2nd count = 34.00±5.63, 3rd count = 30.57±5.14, 4th count = 28.00±4.60), in the *M. viridiflora* treatment group (1st count = 33.00±5.07, 2nd count=28.14±4.47, 3rd count = 25.14±3.98, 4th count=22.14±3.57), in the thyme oil treatment group (1st count=29.57±4.51, 2nd count=25.42±4.24, 3rd count = 22.85±4.14, 4th count = 20.00±3.51), respectively. In the study, the difference between the factors in terms of

Table 3. *Phoretic Varroa negative control group and positive control group pollen trap count results*

Item	Hive No	Day 7	Day 14	Day 21	Day 28
Phoretic *Varroa* negative control group pollen trap count results	1	0	0	1	1
	2	0	0	2	1
	3	0	0	2	1
	4	0	0	2	1
	5	0	0	2	2
	6	0	0	2	2
	7	0	0	2	3
Total		**0**	**0**	**13**	**11**
Positive control group pollen trap count results	1	5	4	5	3
	2	3	6	3	4
	3	4	3	2	5
	4	4	5	4	2
	5	3	5	3	1
	6	3	3	1	3
	7	5	6	3	4
Total		**27**	**32**	**21**	**22**

Henderson-Tilton values is not statistically significant (P>0.05). The difference in pollen trap counts in terms of both factors and counting times was found to be statistically significant (P<0.001).

At each application stage of essential oils, it was checked whether there was a problem with brood cells in the previous application. Dead larvae were not observed in brood cells. Additionally, the inside and surroundings of the hive were carefully examined, and no dead adult honey bees were detected.

Discussion

This study was conducted to find an alternative treatment approach with four essential oils against *V. destructor* in honey bee colonies in Balikesir province, Marmara region of Türkiye. In field studies, thyme oil, *C. verum*, *M. viridiflora*, and *S. aromaticum* oil were impregnated on the strips, they were applied between the frames, three to each hive, four times a week apart, and amitraz was applied to the flight hole by fumigation four times a week apart. Forty-nine hives were divided into seven groups thyme oil, *C. verum*, *M. viridiflora*, *S. aromaticum* essential oil treatment groups, amitraz treatment group, and positive and phoretic *Varroa* negative control group.

The need to minimize or replace synthetic pesticides with natural alternatives has prompted the current seek for environmentally safe treatment options. The plant world has proven to be extremely beneficial, with a wealth of medical resources available to cure a wide range of human and animal ailments. EOs and related monoterpenes have been extensively researched for use in pest management programs [14]. These chemicals have been shown to effectively cure *Ascosphaera apis*, *Paenibacillus larvae*, *Nosema ceranae*, and other honey bee illnesses [21]. When compared to hard acaricides, EOs have been thoroughly tested and have proven effective as miticides against *V. destructor* over time. Extracts obtained from specific botanical species are useful, whereas others are unsuccessful. Presently available for purchase on a global basis are neem products, ryanodine, nicotine, sabadilla, pyrethrins, and rotenone fight against Varroosis [22]. In beekeeping, essential oil-based treatments for controlling *V. destructor* parasites have been licensed for commercialization. For example, Thymovar® (Andermatt BioVet, Grossdietwil, Switzerland) thymol cellulose sponge strips, in addition to ApiLife Var® (Chemicals Laif SPA; Vigonza, Italy) vermiculite tablets that contain eucalyptus oil, camphor, thymol, and, levomenthol, were introduced in Italy. Apiguard® products (Vita Europe Ltd., Basingstoke, England) are patented gels whose unique formulation allows for the gradual release of thymol. Very few herbal-based *Varroa* control products are utilized in beekeeping. Therefore, researchers are continuing studies under both laboratory and field conditions to increase the amount of plant-based products in the fight against *Varroa* in the global market. In one of these studies, one strip of the absorbent paper pad (2x10 cm^2) was dipped in pure rosemary essential oil and placed directly on the outer frames of each hive, with treatments applied at five different rates. The efficacy of rosemary essential oil was found as 93% [23]. Under the same conditions, oils derived from *Thymus satureioides* and *Origanum elongatum* showed comparable efficacy against *V. destructor*. As a result, these oils and their blends, when applied directly to bee hives, have been successfully tested against *Varroa* mites [24]. Camphor has previously been shown to have exceptional bioactivity against *Varroa* mites without being hazardous to bees [25]. In another trial conducted in 5-15 g, 50-150 g, and 20-60 g per liter of air, respectively, thymol, camphor, and menthol killed virtually entirely of the mites without appreciably reducing the bee colony [26]. Also, thymol was absorbed into powder and strips, tested in two different ways. Treatment efficiencies were determined as 96.6% and 92.4%, respectively [17].

S. aromaticum was applied to hives as a fumigant in two different studies. The efficacy was found to be 87% [27] in the first study and 54% [28] in the second study. In another study with *S. aromaticum*, water-soluble protein concentration reduced considerably after the treatments, showing that the mites' metabolism was impaired. Glutathione-S-transferase (GST) bioactivity increased at a low dosage (0.1 µL) but reduced with a greater dosage (1.0 µL), while superoxide dismutase (SOD) and Ca^{2+}-Mg^{2+}-ATPase activities were significantly boosted following treatments. These observations imply that the protective enzyme SOD and detoxifying enzymes Ca^{2+}-Mg^{2+}-ATPase and GST caused the stress response of *V. destructor* to the essential oils and that the detoxifying capacity of *V. destructor* by GST was reduced at higher dosages [29]. *C. verum* [27] and *Laurus nobilis* [28] essential oils were also applied to the hives by fumigation and their treatment efficacy was found to be 52.50% and 75%, respectively.

The effectiveness of nine different essential oils against *Varroa* was evaluated in vitro. According to the results, the activities of bitter melon oil 70%, garlic oil 70%, basil oil 66.6%, thyme oil 61.7%, mustard oil 61.7%, cloves oil 58.3%, eucalyptus oil 55.5%, rosemary oil 53.3%, and mint oil 53.3% were determined [30]. In a study conducted for the effectiveness of thymol, spearmint, tea tree oil and mixture of spearmint and lemongrass essential oils against *Varroa*, the activities were determined as 78.1%, 85.9%, 80.2%, and 84.3%, respectively [31]. The effectiveness against *Varroa* in the *M. viridiflora* treatment group used in our study was determined as 71.3%, and the effectiveness against *Varroa* in the *Melaleuca alternifolia* treatment group mentioned in this study was determined as 80.2%. The reason for this

difference may be the different *Melaleuca* species used, the application method, and geographical and regional differences.

M. viridiflora has previously been used against the American foulbrood agent *Paenibacillus larvae* at a level of 320 mg L^{-1} in laboratory environments and positive results were obtained [29]. In our study, *M. viridiflora* was tested for the first time against Varroosis, and its treatment efficacy was found to be 71.3%. Also, amitraz was used against Varrosis. As a result of this study efficacy was found to be 94.6% [32]. In a study, Apivar and Supatraz, which contain the active ingredient amitraz, were used against *Varroa*. According to the results, it was reported that Supatraz killed the mites faster than Apivar and 90% reduction in the number of *Varroa* mites was observed in 50.9 days for Apivar and 28.4 days for Supatraz [33]. In a study conducted in Türkiye, 98.5% efficacy of amitraz, 96.5% efficacy of flumethrin, and 93.2% efficacy of coumaphos were determined. In this study, it was also reported that the desired effect could not be achieved by fumigation of amitraz. This may be attributed to the acquisition of resistance of *Varroa* agents against amitraz and overlooked points in the use of amitraz [34]. The reasons why different researchers obtained different results in their studies with amitraz are; there may be different companies of the amitraz-containing product used, application methods, application time, and environmental conditions during application. In addition, by placing a special apparatus at the tip of the bee smoker we used in the amitraz fumigation method in field studies, the resulting smoke was completely penetrated into the hive. This increased the success rate we achieved with amitraz in field studies.

During the field studies, minimum-maximum temperature values are 10°C-22°C and average humidity is 67%. These values have created the ideal breeding environment for *Varroa* agents. There are clear discrepancies in the efficiency of the Varroosis treatment between the results of our field trial and the articles cited above. There may be many reasons for this situation. The most important of these reasons is that the contents of essential oils vary depending on the season and region. Furthermore, the efficacy of particular components is determined by the evaporation pressure in hives, which varies depending on the time of year and the surrounding humidity and temperature during treatment application.

To sum up, essential oils can be used safely against the phoretic cycle of *Varroa* agents in temperature ranges (10-22°C) in October, which is the beginning of the winter period. In this way, the use of chemical active substances can be prevented by ensuring that honey bee colonies enter the winter season with less *Varroa* load. In this way, honey bee colonies emerge healthier and stronger in the spring.

As a result, according to the findings obtained from our field study, it has been revealed that essential oils can be used instead of chemicals against *Varroa*. More laboratory and field studies are needed in the fight against Varroosis with essential oils. With these studies, essential oils will become more standard and reliable.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available from the corresponding author (M. Özüiçli) on reasonable request.

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Competing Interest: The author reports no declarations of interest.

Author Contributions: MÖ planned the study and designed the experiments, YB planned and performed the data analyses. The final version of the manuscript was read and approved by all authors.

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Research Article

Distribution of Species and Biotypes of *Brucella* Isolates Obtained from Sheep and Cattle Abortions

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Abstract

This study was carried out to evaluate *Brucella* spp. isolated from various tissue samples of aborted sheep and bovine fetuses sent to the laboratory of Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University between 2011 and 2023 years and determine the *Brucella* species and biotype diversity that carry a higher risk for abortion complications in these animals. In this context, 155 *Brucella* spp. isolates obtained from aborted fetuses were identified by species-specific Bruce-ladder PCR and biotyped using conventional biotyping methods. As a result of the study, *B. melitensis* and *B. abortus* were identified in 92.5% (n=74) and 7.5% (n=6) of sheep, *B. abortus* and *B. melitensis* were identified in 80% (n=60) and 20% (n=15) of cattle, respectively. *B. melitensis* biotype 2 in sheep and *B. abortus* biotype 3 in cattle were found as the dominant biotypes in these definitive hosts. In the Kars region, where brucellosis is endemic, while the biotype responsible for cattle brucellosis (*B. abortus* biotype 3) maintained its dominance over a 20-year period, there is a profile change from *B. melitensis* biotype 3 to *B. melitensis* biotype 2 in sheep. Considering the period covered by the study and the sample size analyzed, the data obtained provide up-to-date and important information about *Brucella* species and biotypes in Kars region and the animal species that host these agents.

Keywords: Sheep, Cattle, *Brucella melitensis*, *Brucella abortus*, Biotype, Bruce-ladder multiplex PCR

Introduction

Brucellosis is a prevalent zoonotic bacterial disease worldwide, affecting a wide range of mammals, including humans [1]. In animals, brucellosis manifests with various clinical presentations such as abortion, infertility, retained placenta, orchitis, epididymitis and, rarely, arthritis [2]. This often results in severe economic losses, including reduced reproductive productivity of livestock and animal replacement costs associated with removal from the herd [3]. Human infections are associated with direct contact with infected animals or their products and ingestion of contaminated dairy products, particularly cheese and unpasteurized milk, and exposure to infectious aerosols. Human brucellosis is rarely fatal, but it significantly affects various body systems (reproductive, musculoskeletal, central nervous, etc.) and causes severe and sometimes permanent sequelae, including disability [4].

The etiological agent of brucellosis is a non-motile, non-capsulated, non-spore-forming, facultative intracellular Gram-negative coccobacilli [5]. To date, in the *Brucella* genus there are 12 closely related species have been described based on both genetic and immunological characteristics [6,7]. Members of the *Brucella* genus have strict host preferences, but recent adaptations of the classical *Brucella* species to new hosts have been remarkable. Among these, *Brucella abortus* and *Brucella melitensis* are included in the classical species by primarily effecting cattle and sheep/goats, respectively [2,7-9]. In modern *Brucella* systematics, *B. abortus* and *B. melitensis* are divided into some biotypes. *B. melitensis* has three biotypes (biotype 1-3) and *B. abortus* has 7 biotypes (biotype 1-6 and 9) [5]. Although both *Brucella* species have a preferred host, they can cause infection in more than one host through their different biotypes [10]. The characterisation and biotyping of *Brucella* species are

crucial at various stages such as molecular epidemiological studies, determining the geographical origin of the source of infection and determining the relationships between isolates [11], development of control and eradication policies following epidemiological studies and even monitoring and controlling the efficacy of vaccine strains [10]. Periodic monitoring of species and biotype distribution and taking an inventory every ten years would make it more feasible to implement in the areas mentioned above.

With the advances in molecular techniques and a better understanding of the genomes of various *Brucella* species, molecular methods based on PCR are widely used in both differential diagnosis and molecular epidemiologic studies [12]. In this context, multiplex PCR techniques such as Bruce-Ladder [13], have been developed that can identify *Brucella* isolates down to strain level and distinguish field strains from vaccine strains. However, the same principled methodology is still unable to distinguish *Brucella* biotypes. Biotyping of *Brucella* strains is still based on some characteristics of the isolates such as CO_2 requirement, H_2S production, urea hydrolysis, basic fuchsin and thionine sensitivity, and phage susceptibility, as well as agglutination with monospecific sera, and lysis by *Brucella* phages [14,15].

This study aimed to determine the *Brucella* species and biotypes distribution in clinical brucellosis cases in farm animals.

Material and Methods

Ethical Statement

This study vas approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: KAÜ-HADYEK/2020/103).

Reference *Brucella* Strains

In the study, *B. abortus* 544, *B. melitensis* 16 M, *B. abortus* S19, *B. melitensis* Rev 1, *B. ovis* 63/290 strains belong to the culture collection kept at Department of Microbiology, Faculty of Veterinary Medicine of Kafkas University, Türkiye were used as reference *Brucella* strains.

Brucella Field Isolates

The study material was consisted of 155 *Brucella* isolates obtained from aborted sheep and cattle fetuses. *Brucella* isolates were obtained from samples such as lung, liver and abomasum contents of aborted fetuses sent to Kafkas University, Faculty of Veterinary Medicine, Department of Microbiology between 2011 and 2023.

Tissue and organ samples of aborted fetuses were cultured on blood agar containing 5-10% sheep blood. Double cultivation was performed for all samples and incubations were performed in aerobic and 5-10% CO_2 environment at 37°C for 5-7 days.

Colony morphology, Gram staining and growth characteristics, catalase, oxidase and urease test results were considered to identify the isolates in *Brucella* genus level [2]. Isolates previously isolated and identified as *Brucella* spp. were stored in *Brucella* Broth with 20% glycerin and stored at -80°C until the molecular identification and biotyping tests performed.

Species-Specific Identification of *Brucella* Isolates by PCR

- *Genomic DNA Extraction*

For the chromosomal DNA extraction, the single cell lysis buffer (SCLB) method was performed from fresh cultures of the *Brucella* isolates [16].

- *Bruce-Ladder Multiplex PCR*

Species-specific identification of the *Brucella* isolates was performed with Bruce-ladder PCR, a multiplex PCR [13]. The primers and target genes used in the Bruce-Ladder PCR are given in *Table 1*. Bruce-ladder multiplex PCR was consisted of 5 µL 5xLongAmp™ Taq Reaction Buffer, 1 µL of $MgCl_2$ (20 mM), 0.75 µL of dNTP (10 mM), 1 µL of each primer (12 pieces) (20 pmol), 1 µL of LongAmpR Taq DNA Polymerase (5 U) and 3 µL of template DNA (50 ng/µL). The thermal condition of Bruce-ladder PCR was set with an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 35 sec, primer binding at 62°C for 45 sec, elongation at 65°C for 3 min and a final elongation at 65°C for 10 min. Amplified products were analysed on 1.5% agarose gel. The band sizes obtained were evaluated and *Brucella* isolates were identified at species-level considering the band combination in *Table 1*.

Biotyping of *Brucella* Isolates

Biotyping of the *Brucella* isolates were performed based on H_2S production, growth in the presence of thionine and basic fuchsin dyes, and agglutination reactions with mono-specific A and M anti-sera [14,15,17]. Tryptic soy agar (TSA) (Merck, 105458) supplemented with dextrose and heat inactivated horse serum solution (5%) and was employed as the basal medium for all cultural examinations. Inoculated these plates were incubated at 37°C in condiditions with 5-10% CO_2 for 4-5 days [9].

- *CO_2 Requirement and H_2S Production*

CO_2 requirement for growth was evaluated in first isolation of strains. For H_2S production test, paper strips with lead acetate were placed between the tube edge and the screw cap so that they did not come into contact with the TSA medium. For each *Brucella* isolate, inoculation was incubated in 5-10% CO_2 at 37°C for 4-5 days. At the end of the incubation period, the results were evaluated according to the color change in the lead acetate papers [14,15].

Table 1. *List of primer pairs used for Bruce-ladder multiplex PCR and the reaction results of the Brucella species*

Primer	Sequence (5'–3')	Amplicon size (bp)	DNA targets	Strains
BMEI0998f	ATCCTATTGCCCCGATAAGG	1682	Glycosyltransferase, gene *wboA*	*B. abortus* *B. melitensis* *B. suis* *B. canis* *B. abortus* S19 *B. melitensis* Rev 1
BMEI0997r	GCTTCGCATTTTCACTGTAGC			
BMEII0843f	TTTACACAGGCAATCCAGCA	1071	Outer membrane protein, gene *omp31*	*B. melitensis* *B. ovis* *B. suis* *B. canis* *B. melitensis* Rev 1
BMEII0844r	GCGTCCAGTTGTTGTTGATG			
BMEI1436f	ACGCAGACGACCTTCGGTAT	794	Polysaccharide deacetylase	*B. abortus* *B. melitensis* *B. ovis* *B. suis* *B. abortus* S19 *B. melitensis* Rev 1
BMEI1435r	TTTATCCATCGCCCTGTCAC			
BMEII0428f	GCCGCTATTATGTGGACTGG	587	Erythritol catabolism, gene *eryC* (D-erythrulose-1-phosphate dehydrogenase)	*B. abortus* *B. melitensis* *B. ovis* *B. suis* *B. canis* *B. melitensis* Rev 1
BMEII0428r	AATGACTTCACGGTCGTTCG			
BMEI0535f	GCGCATTCTTCGGTTATGAA	450	Immunodominant antigen, gene *bp26*	*B. abortus* *B. melitensis* *B. ovis* *B. suis* *B. canis* *B. abortus* S19 *B. melitensis* Rev 1
BMEI0535r	CGCAGGCGAAAACAGCTATAA			
BR0953f	GGAACACTACGCCACCTTGT	272	ABC transporter binding protein	*B. suis* *B. canis*
BR0953r	GATGGAGCAAACGCTGAAG			
BMEI0752f	CAGGCAAACCCTCAGAAGC	218	Ribosomal protein S12, gene *rpsL*	*B. melitensis* Rev 1
BMEI0752r	GATGTGGTAACGCACACCAA			
BMEII0987f	CGCAGACAGTGACCATCAAA	152	Transcriptional regulator, CRP family	*B. abortus* *B. melitensis* *B. ovis* *B. suis* *B. canis* *B. abortus* S19 *B. melitensis* Rev 1
BMEII0987r	GTATTCAGCCCCCGTTACCT			

- Growth in the Presence of Thionine and Basic Fuchsin Dyes

Brucella colonies cultivated freshly on TSA supplemented with dextrose and heat inactivated horse serum solution were collected with peptone-saline from the agar surface, and a bacterial inoculum was prepared containing approximately 1x10^9 CFU/mL bacteria via McFarland standard 4.

Inoculations were done with a steril swab from suspansion of field isolates and standard strains onto TSA slides containing thionine and basic fuchsin (20 µg/mL) As the expression is the same, it would be sufficient to use only one of them. Slides were incubated in at 37°C 5-10% CO_2 for 4-5 days. The results were evaluated according to their growth status [10,14].

- Agglutination with Mono-Specific Anti-Sera (A and M)

A loopful of bacterial colony was taken from the fresh culture of each isolates to be evaluated and a bacterial suspension was prepared in 0.25 mL of physiological saline. One drop of each monospecific antisera A and M were placed on a clean slide and one drop of bacterial suspension was added and mixed throughly. The results were analysed according to the agglutination within one minute [14,15].

Statistical Analysis

The Pearson Chi Square test, one of the nonparametric tests, was used to measure changes in the isolation rates of the obtained *B. abortus* and *B. melitensis* biotypes according to years and animal groups.

Results

Bruce-Ladder Multiplex PCR Results

As a result of the Bruce-ladder PCR, 74 (92.5%) of the *Brucella* isolates from sheep originated were identified as *B. melitensis* and 6 (7.5%) as *B. abortus*, while 60 (80%) of the *Brucella* isolates from cattle originated were identified as *B. abortus* and 15 (20%) as *B. melitensis*. It was observed that all of the *Brucella* isolates identified were field strains.

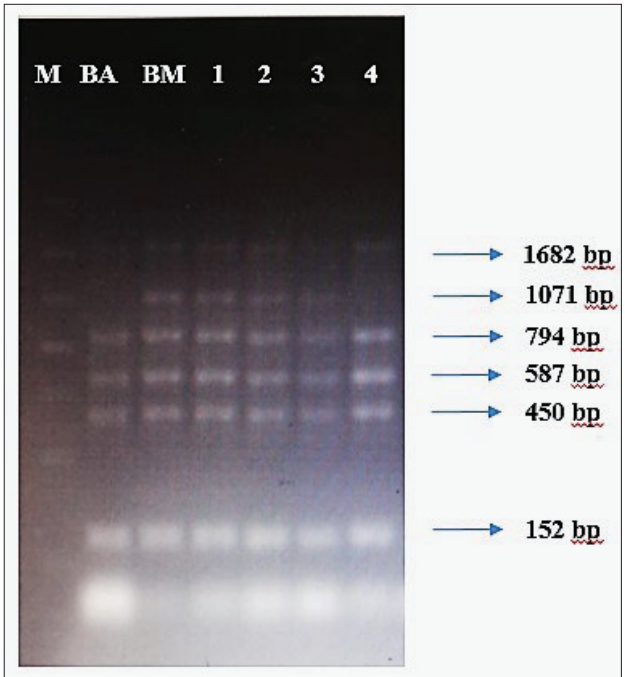

Fig 1. 1.5% agarose gel electrophoresis images of Bruce-ladder PCR products. M: GeneRuler 1 kb DNA Ladder (Thermo Sci SM0311). BA: *B. abortus* biotype 1 (544), BM: *B. melitensis* biotype 1 (16M), 1-3: Field strain (*B. melitensis*), 4: Field strain *(B. abortus)*

Fig. 1 shows the agarose gel electrophoresis analysis of the amplified products of the *Brucella* strains identified by Bruce-ladder multiplex PCR.

Biotyping Results

In the present study, a total of 155 *Brucella* isolates (80 from sheep and 75 from cattle) were biotyped using conventional methods. Out of the 74 (92.5%) *B. melitensis* isolates obtained from sheep, 43 (58.1%) were biotyped as *B. melitensis* biotype 2, 21 (28.4%) as *B. melitensis* biotype 3 and 10 (13.5%) as *B. melitensis* biotype 1. Among the 6 *B. abortus* isolates obtained from sheep, 3 (50%) were biotyped as *B. abortus* biotype 1 and 3 (50%) as *B. abortus* biotype 3 *(Table 2)*.

Out of the 60 *B. abortus* isolates obtained from cattle, 29 (48.33%) were biotyped as *B. abortus* biotype 3, 23 (38.33%) as *B. abortus* biotype 1, and 8 (13.33%) as *B. abortus* biotype 2. Among the 15 cattle *B. melitensis* isolates, 10 (67%) were biotyped as *B. melitensis* biotype 3, 3 (20%) as *B. melitensis* biotype 2, and 2 (13%) as *B. melitensis* biotype 1 *(Table 2)*.

In Kars province, given the isolation rates of biotypes according to year, it is seen that among the sheep-originated strains, *B. melitensis* biotype 3 was predominant in 2004-2006 *(Table 3)*, 2015, 2019, 2021, and 2023 *(Table 4)*, and *B. melitensis* biotype 1 in 2011, 2014, and 2016 *(Table 4)*. Interestingly *B. melitensis* biotype 2 was dominant in 2012, 2017, 2018, 2020 and 2022 in sheep *(Table 4)*. And all these *B. melitensis* biotypes were found in 2013 and 2016 *(Table 4)*. When analyzing the strain distributions in five-year periods, it is seen that *B. melitensis* biotype 2 maintained its presence between 2011-2015 and 2016-2020, and *B. melitensis* biotype 2 and biotype 3 maintained their presence between 2021-2023 *(Table 4)*.

B. abortus biotype 3 was the dominant biotype in cattle in 1998-2010 *(Table 3)* and in 2011, 2012, 2016-2018 and 2022 *(Table 5)*, whereas, *B. abortus* biotype 1 was the dominant biotype in 1998-2002 *(Table 3)* and 2013, 2018, 2020-2023 *(Table 5)*. *B. abortus* biotype 2 was the only biotype isolated in 2019 *(Table 5)*. Therefore, as a result

Table 2. *Properties and distribution of B. melitensis and B. abortus biotypes*

Biotype	Characteristics						Origins		P Value
	CO_2^*	H_2S^{**}	BF	T	A	M	Sheep (%)	Cattle (%)	
B. melitensis biotype 1	-	-	+	+	-	+	10 (13.5)	2 (13)	0.022
B. melitensis biotype 2	-	-	+	+	+	-	43 (58.1)	3 (20)	0.000
B. melitensis biotype 3	-	-	+	+	+	+	21 (28.4)	10 (67)	0.000
B. abortus biotype 1	+	+	+	-	+	-	3 (50)	23 (38.33)	0.000
B. abortus biotype 2	+	+	-	-	+	-	0 (0)	8 (13.3)	0.000
B. abortus biotype 3	+	+	+	+	+	-	3 (50)	29 (48.33)	0.000

** CO_2 requirement, ** H_2S Production, **BF:** Growth in Basic Fuchsin, **T:** Growth in Thionin, **A:** Aglutination with Antisera A, **M:** Aglutination with Antisera M*

Table 3. *Results obtained from biotyping studies for sheep-cattle brucellosis in Kars province*

Year	Origin	*B. melitensis* biotype 1 (n, %)	*B. melitensis* biotype 2 (n, %)	*B. melitensis* biotype 3 (n, %)	*B. abortus* biotype 1 (n, %)	*B. abortus* biotype 2 (n, %)	*B. abortus* biotype 3 (n, %)	Total Number of the Isolate	Reference
2004-2006	Sheep	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	0 (0)	25	[18]
2011-2023		10 (12.5)	43 (53.75)	21 (26.25)	3 (3.75)	0 (0)	3 (3.75)	80	Present study
1998-2002	Cattle	0 (0)	0 (0)	0 (0)	13 (46.43)	0 (0)	15 (53.57)	28	[19]
1998-2002		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	25 (100)	25	[20]
2001-2006		0 (0)	0 (0)	0 (0)	3 (6.25)	0 (0)	45 (93.75)	48	[21]
2008		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	16 (100)	16	[22]
2009-2010		0 (0)	0 (0)	1 (3.2)	0 (0)	0 (0)	30 (96.8)	31	[23]
2008-2010		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	25 (100)	25	[24]
2011-2023		2 (2.7)	3 (4)	10 (13.3)	23 (30.7)	8 (10.6)	29 (38.7)	75	Present study

Table 4. *Distribution of identified biotypes in sheep in the study according to years*

Year	The Number of Isolates	Identified Biotypes					
		B. melitensis biotype 1	*B. melitensis* biotype 2	*B. melitensis* biotype 3	*B. abortus* biotype 1	*B. abortus* biotype 2	*B. abortus* biotype 3
2011	13	6	5	1	0	0	1
2012	11	1	9	1	0	0	0
2013	7	2	2	2	1	0	0
2014	4	0	0	1	2	0	1
2015	2	0	0	2	0	0	0
2016	3	1	1	1	0	0	0
2017	10	0	8	2	0	0	0
2018	1	0	1	0	0	0	0
2019	1	0	0	1	0	0	0
2020	13	0	10	3	0	0	0
2021	7	0	1	6	0	0	0
2022	7	0	6	0	0	0	1
2023	1	0	0	1	0	0	0

Table 5. *Distribution of identified biotypes in cattle in the study according to years*

Year	The Number of Isolates	Identified Biotypes					
		B. abortus biotype 1	*B. abortus* biotype 2	*B. abortus* biotype 3	*B. melitensis* biotype 1	*B. melitensis* biotype 2	*B. melitensis* biotype 3
2011	8	0	0	5	1	0	2
2012	7	2	0	5	0	0	0
2013	4	3	0	1	0	0	0
2015	4	0	0	0	0	1	3
2016	7	0	0	6	0	0	1
2017	5	0	0	5	0	0	0
2018	8	4	0	3	0	0	1
2019	8	1	4	1	0	1	1
2020	12	3	4	2	1	0	2
2021	7	6	0	0	0	1	0
2022	2	1	0	1	0	0	0
2023	3	3	0	0	0	0	0

of the Chi-Square analysis (χ^2 = 154.362; P=0.000), it was seen that there was a statistically significant difference in the diversity of biotype isolation according to years.

Discussion

Brucellosis is recognized as one of the global zoonoses that causes significant economic losses in the livestock industry worldwide and poses a serious public health problem [25]. Animal brucellosis is mainly characterized by reproductive system diseases [25], resulting in some clinical signs such as placental retention, birth of weak offspring, dead offspring, infertility and abortion [26]. In livestock, herd identification of brucellosis is usually based on the typical clinical signs, especially serological examinations and isolation of the causative agent. In any case, the diagnosis of brucellosis in one or more infected animals indicates the infection in a population. Therefore, detecting the causative agent in species or biotype based is extremely important in supporting the rapid and accurate diagnosis of Brucellosis and critical in the control and eradication of the infection in livestock [27].

Direct laboratory diagnostic methods of brucellosis in animals, such as bacterial isolation, have high specificity, but are time-consuming and require an appropriate degree of biosecurity. PCR-based molecular methods have been reported as an effective tool for rapid detection and confirmation of *Brucella* infection as well as for differentiation of *Brucella* species. Both culture and molecular techniques can definitively demonstrate the presence of infection and are effective in identifying the responsible *Brucella* species [28]. It has been emphasized that a molecular technique such as Bruce-ladder, which has been shown to work particularly well on *Brucella* spp. DNA and therefore will not be adversely affected by non-target DNA in contaminated agents, is a powerful technique both in the diagnosis of *Brucella* agents and in the differentiation of field and vaccine strains [9]. So, in the present study, Bruce-ladder multiplex PCR technique which has the discrimination power including vaccine strains, was applied to confirm the identification of the isolates obtained at the species level. The isolates obtained from sheep and cattle were identified as *B. abortus* and *B. melitensis*. All isolates were found to be field strains.

Brucella melitensis and *B. abortus* are predominant agents for small ruminants and cattle, respectively. Although a very strict host preference is observed in the *Brucella* species, the genus members have a wide host diversity [23]. These species are among the pathogenic bacteria that tend to adapt to new hosts and can be transmitted naturally to their primary hosts through direct or indirect ckontact and sometimes incidentally to other susceptible hosts [29]. While cross-infections between species were once rare, they have now become almost commonplace [12]. This situation has been reported in many studies. Abortions associated with *B. abortus* in sheep [30-33] and *B. melitensis* in cattle [30,32,34] have been widely reported. This situation has often been interpreted as the possibility that animals may be more likely to be exposed to *Brucella* infection, especially those originating from different species of agents, due to the coexistence of different animal species [9,10,12,26]. However, it was emphasized that this situation may be accompanied by factors such as the infectivity of the pathogenic agent, the immune structure of the host and the structure of the animal population [9]. In the present study, 20% (15/75) of the cattle-originated *Brucella* strains were identified as *B. melitensis* and 7.5% (6/80) of the sheep-originated *Brucella* strains were identified as *B. abortus*. These results, as in the aforementioned studies, show that the dominant species circulating in livestock can overcome host species barriers and adapt to new hosts despite their known host preferences. This is also an indication that in the Kars region, where Brucellosis is frequently seen and livestock farming is intensive, cattle and sheep are usually kept together and it is inevitable that these animals are exposed to the different *Brucella* species and the emergence of the cross-infection is inevitable. Therefore, the identification of the species involved in animal brucellosis, their potential to adapt to new environments and the changes that may occur in their epidemiological characteristics can be considered as effective factors to be taken into consideration in the design of protection and control programs to solve the complexity of the interactions of these microorganisms with each other as well as their interactions with animals and humans.

It has been revealed that different biotypes are effective in bovine brucellosis in many countries of the World. In Egypt [1], Tanzania [6], Italy [8], Yemen [17], Bangladesh [31], Iran [32], South Africa [34], many West African countries [35], *B. abortus* biotype 3 was reported as the dominant biotype in cattle, whereas, *B. abortus* biotype 1 was dominant in some Latin American countries [36], Zimbabwe [37] and Brazil [38]. *B. abortus* biotype 3 is the dominant biotype in bovine brucellosis in Türkiye, as well. Sarısayın et al.[39] identified 87.93%, 7.76% and 0.86% of bovine *Brucella* isolates as *B. abortus* biotype 3, *B. abortus* biotype 1 and *B. abortus* biotype 2, respectively. Şahin et al.[21] identified 93.75% and 6.25% of which originated aborted bovine foetus isolates as *B. abortus* biotype 3 and biyotype 1, respectively. Büyükcangaz and Şen [40] identified *B. abortus* biotype 3 from bovine aborted fetuses at a rate of 87.5%. Büyük and Şahin [23] reported that the dominant biotype was *B. abortus* biotype 3 in milk and aborted bovine fetus samples. Gürbilek et al.[10] identified 96% of the isolates originated bovine as *B. abortus* biotype 3 in their studies on biotyping of 114 *Brucella* isolates different originated.

Erdenliğ Gürbilek et al.[9] identified 94.2% of the *Brucella* spp. isolated from cattle in 2009-2011 as *B. abortus* biotype 3. All isolates recovered from bovine originated samples were identified as *B. abortus* biotype 3 in other studies [31,41-44]. In the present study, it was observed that *B. abortus* biotype 3 was the predominant biotype in bovine *Brucella* isolates in accordance with the aforementioned studies. This shows that the causative species and biotype have not changed in bovine abortions and *B. abortus* biotype 3 is still the predominant in bovine brucellosis in our country and Kars province. However, Sözmen et al.[20] reported 81.8% and 18.2% of the isolates as *B. abortus* biotype 1 and *B. abortus* biotype 3, respectively. In the present study, 38.33% of *B. abortus* isolates were identified as *B. abortus* biotype 1. For this reason, it is thought to be important in terms of evaluating the agent as the second dominant species after *B. abortus* biotype 3. This situation also shows that the importance of species and biotype differences in the occurrence of the disease should be taken into consideration.

When sheep brucellosis is evaluated in various countries of the world, variability is observed between biotypes. Behroozikhah et al.[25], found 92.8% and 6.8% of the isolates as *B. melitensis* biotype 1 and *B. melitensis* biotype 2 in 2007-2009 in Iran. In Northern Cyprus, Demirpençe et al. [7], reported that the dominant biotype was *B. melitensis* biotype 1 followed by *B. melitensis* biotype 3. In addition, in Iran, Dadar and Alamian [45] reported that *B. melitensis* biotype 1 was dominant in sheep abortions, followed by *B. melitensis* biotype 2 and biotype 3 in 2016-2019. In Yemen, Al-Afifi et al.[17], isolated *B. melitensis* biotype 3, followed by *B. melitensis* biotype 2 in sheep. Various studies have been conducted on the biotype distribution of *B. melitensis* in Türkiye and *B. melitensis* biotype 3 was found the dominant biotype [9,10,30,42,44,46-48]. However, there are some studies reporting predominancy of *B. melitensis* biotype 1 and biotype 2 as well as *B. melitensis* biotype 3. Sarısayın et al.[39] identified 78% of sheep *Brucella* isolates as *B. melitensis* biotype 2 and 22% as *B. melitensis* biotype 1. In the present study, *B. melitensis* biotype 2 was the dominant biotype isolated from sheep abortions with a rate of 58.1%. However, this agent was followed by *B. melitensis* biotype 3 with 28.4% and *B. melitensis* biotype 1 with 13.5%. It is thought that this result may be due to the fact that the prevalence of *B. melitensis* biotype 2 started to increase and thus became dominant in the Kars region. In addition, factors such as the year intervals in which the studies were conducted, the difference in the origin of the isolates of the geographical regions, or the emergence of some variant or atypical strains may be effective in obtaining different results. Therefore, it is considered to be of great importance to periodically determine the *Brucella* species and biotypes causing brucellosis in our country. In addition, similar to the present study, Refai [49] reported that the dominant biotype isolated from sheep and goats was *B. melitensis* biotype 2 in a study covering a 10-year period in Saudi Arabia.

In a province-based evaluation of cattle-sheep brucellosis, the dominant biotypes obtained since the first biotyping studies conducted in the Kars region have been reported as *B. abortus* biotype 3 and followed by *B. abortus* biotype 1 in cattle, and *B. melitensis* biotype 3 in sheep. Indeed, Genç and Kamber [19], reported the isolates they detected in cattle in 1998-2000 as *B. abortus* biotype 3 and following *B. abortus* biotype 1. Sözmen et al.[20], identified all strains as *B. abortus* biotype 3 in 1998 and 2002. Şahin et al.[21] typed the isolates as *B. abortus* biotype 3 and following *B. abortus* biotype 1 in 2001-2006. Beytut et al.[41], isolated and identified *B. abortus* biotype 3 from cows with a history of abortion due to *B. abortus* in the winter season of 2002. Çelebi and Otlu [22] typed all of the isolates as *B. abortus* biotype 3 in 2008 and Büyük and Şahin [23], identified the isolates as *B. abortus* biotype 3 (96.77%), *B. abortus* biotype 6 (4.87%), *B. abortus* biotype 9 (3.22%), *B. abortus* biotype 1 (2.43%) in 2009-2010. Dağ et al.[24], bio-typed all isolates as *B. abortus* biotype 3 in 2008-2010. When looking at the studies covering a 14-year period, it is seen that there is a stable situation in dominancy of biotype profile which is primarily *B. abortus* biotype 3 followed by biotype 1. However, different biotypes such as *B. abortus* biotype 6 and biotype 9 have been scarcely identified as abortive agents. Biotyping studies on sheep brucellosis in the Kars region are very limited. Şahin et al.[18], biotyped all isolates as *B. melitensis* biotype 3 in a study conducted in 2004-2006. In the current study covering the years 2011-2023, out of the 60 *B. abortus* isolates obtained from cattle, 29 (48.33%) were biotyped as *B. abortus* biotype 3, 23 (38.33%) as *B. abortus* biotype 3, 23 (38.33%) as *B. abortus* biotype 1, and 8 (13.33%) as *B. abortus* biotype 2. This result shows that the agent responsible for clinical brucellosis in cattle has not changed and is still *B. abortus* biotype 3, followed by *B. abortus* biotype 1. In sheep out of the 74 (92.5%) *B. melitensis* isolates obtained from sheep, 43 (58.1%) were biotyped as *B. melitensis* biotype 2, 21 (28.4%) as *B. melitensis* biotype 3 and 10 (13.5%) as *B. melitensis* biotype 1. In sheep, unlike the aforementioned study, *B. melitensis* biotype 2 became dominant and its prevalence started to increase. This may be due to the fact that the species and biotypes are responsible for brucellosis between regions within a country and even between flocks. And, uncontrolled animal movements can be thought of as a contributing factor.

In conclusion in light of the findings of this study and previous studies in this direction, it is understood that the most common biotypes in our country continue to be *B. abortus* 3 followed by *B. abortus* biotype 1 in cattle, but

unlike sheep, the prevalence of *B. melitensis* biotype 2 has started to increase and become the dominant biotype. Considering the period covered by this study (13 years) and the sample size analyzed, the data obtained provide up-to-date and important general information on *Brucella* species and biotypes in the Kars region and the animal species prone to exposure with these agents.

Declarations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (E. Çelik).

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Ethical Statement: This study vas approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: KAÜ-HADYEK/2020/103).

Conflict of Interest: The authors declare no conflict of interest.

Author Contributions: EÇ: Design of study; DNA extraction, PCR analysis and biotyping of isolates, writing – review & editing; AGS: Design of study, PCR analysis and biotyping of isolates; FB: Design of study, evaluation the results, review; SO: Design of study, evaluation the results, review; MŞ: Design of study, evaluation the results, review; ÖÇ: Design of study, evaluation the results; MRC: PCR analysis of isolates; SD: DNA extraction and biotyping of isolates; EB: DNA extraction of isolates; YE: DNA extraction and biotyping of isolates.

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Research Article

Antimicrobial and Antibiofilm Effects of Melittin and Apamin Bee Venoms from *Apis mellifera* L. on ESKAPE Pathogens and Cytotoxic Effects on L929 Fibroblast Cells

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Abstract

Public health is facing a worldwide challenge due to the emergence of multiple antibiotic resistance in ESKAPE *(Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Escherichia coli*) infections. The natural antimicrobial nature of bee venoms makes them promising antimicrobial candidates against antibiotic-resistant pathogens. In this study, melittin and apamin bee venoms were investigated to generate novel antimicrobial agents effective against ESKAPE microorganisms. The antimicrobial and antibiofilm activities of melittin and apamin synthesized from endemic *Apis mellifera* L. bee venom were tested against ESKAPE microorganisms using minimal inhibition, minimal bactericidal inhibition and time-kill curve methods. The in-vitro cytotoxicity effect of melittin and apamin for L929 fibroblast cells was also examined. Synthesized melittin and apamin (0.78-600 µg/mL) were antimicrobial against ESKAPE pathogens. Time-kill curve studies confirmed the growth retardation effect and bactericidal activity of melittin and apamin. Antibiofilm studies also showed that melittin and apamin significantly (melittin 8%-82%, apamin 1.8%-78%) inhibited the biofilm formed by ESKAPE pathogens. Melittin and apamin were not cytotoxic to L929 fibroblast cells. We found that melittin and apamin have high antibacterial properties. These naturally synthesized bee venoms offer a promising powerful solution to fight various pathogens.

Keywords: ESKAPE, Bee venom, Melittin, Apamin, Antimicrobial, Antibiofilm, Time-kill curve

Introduction

The growing effect that antimicrobial resistance (AMR) is having on morbidity, mortality, and healthcare expenditures makes it one of the most alarming public health issues in the world. AMR is linked to nearly 700.000 fatalities annually [1,2]. Antibiotic-resistant bacteria are predicted to be responsible for over 33.000 fatalities in the European Union (EU) by the year 2050 [3]. In spite of efforts to combat antimicrobial resistance (AMR), the overuse of antibiotics, particularly in developing nations where this practice permits the issuance of unnecessary prescriptions and the distribution of antibiotics to uninvited patients, the ongoing use of antibiotics, self-medication without consulting a doctor, and contact with nosocomial infections have resulted in the emergence of multidrug-resistant (MDR) bacteria. These bacteria account for approximately fifteen and a half percent of hospital-acquired infections (HAI) worldwide [4-7]. Among these pathogens, six species were considered particularly threatening due to their potential mechanisms and pathogenicity. ***E**nterococcus faecalis*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, and ***E**scherichia coli* are together referred to as the ESKAPE pathogens [8]. ESKAPE pathogens, which contribute significantly to the disease burden of countries and are associated with life-threatening bacteraemia, urinary tract infections, pneumonia, meningitis and wound infections, especially

in intensive care units, and are frequently isolated from clinical settings, are one of the leading causes of mortality and morbidity worldwide [9].

Antimicrobial peptides (AMPs) are cationic amphipathic peptides that are naturally occurring antibacterial compounds that are employed as one of the preferred antimicrobial groups in the therapy of infectious disorders due to the issue of antibiotic resistance. It has been demonstrated that AMPs' potential potency, quick action, and ability to eradicate both Gram-positive and Gram-negative bacteria have drawn interest in them as supplemental or alternative antibiotics [10]. Melittin is an alpha-helical peptide that belongs to the class of amphipathic polar molecules (AMPs). Its chemical formula is C131H228N38O32. The venom of *Apis mellifera*, the species of European honey bees, contains a water-soluble poisonous peptide called melettin, which makes about 52% of the dry weight of the venom [11]. This 26-amino acid peptide has a high surface activity. Its antimicrobial activity is thought to be due to its hydrophobic amino acids in the N-terminal and middle region (residues 1-20) and cationic amino acids in the C-terminal region (residues 21-24). Melittin is not only a potent antimicrobial but also a powerful anti-inflammatory and anticancer agent, and at low doses it increases cell growth and proliferation [12,13].

Apamin is a small peptide component derived from *Apis mellifera* [14]. Essential structure assurance appeared that apamin contains 18 amino corrosive buildups, with two intramolecular disulfide bonds (Cys1-Cys11 and Cys3-Cys15). In addition, the C-terminal buildup is amidated; this post-translational adjustment is common for peptide poisons from creature venoms [15]. Apamin has long been known as a particularly specific blocker of little conductance Ca^{2+}-activated K^+ (SK) channels [16], subsequently, it acts as an allosteric inhibitor [17]. Apamin is known to have antimicrobial activity among its pharmacological effect [18-20]. The aim of this study was to investigate the antibacterial and antibiofilm activity of melittin and apamin components from *Apis mellifera* L. on ESKAPE pathogens, important hospital pathogens, and their cytotoxic effect on L929 fibroblast cells.

Material and Methods

Ethical Statement

Ethics committee approval is not required for this study.

Isolation of Melittin and Apamin from *Apis mellifera* L. Bee Benom

Melittin and apamin used in the study were obtained from Çetinkaya [21]. Çetinkaya [21] isolated melittin and apamin from *Apis mellifera* L. bees in the bee pasture in Kütahya Dumlupınar University Evliya Çelebi Campus.

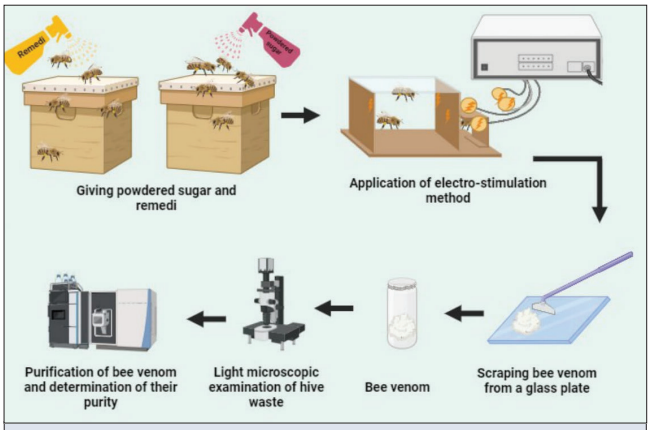

Fig 1. Bee venom collection scheme and process flow. CFU: colony-forming units

They sprayed using remediation, which encourages bees to avoid chemical-synthetic pesticides and prefer organic farming methods. They collected bee venoms by electro-stimulation as shown in *Fig. 1*, and measured their purity by reverse phase high pressure liquid chromatography (RP-HPLC) [21,22].

Antimicrobial and Antibiofilm Activity of Melittin and Apamin Components on ESKAPE Pathogens

- Bacterial Strains

E. faecalis American Type Culture Collection (ATCC) 29212, *S. aureus* ATCC 29213, *K. pneumoniae* ATCC 700603, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 strains were used in this study. These standard strains stored at -80°C were inoculated on tryptic soy agar (TSA, Neogen®, Lansing, MI, USA) and incubated overnight at 37°C in an incubator. After incubation, bacterial suspensions with 0.5 McFarland turbidity were prepared [24].

- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

To determine the MIC values of antimicrobial agents by liquid microdilution method, 100 µL of cation added Mueller Hinton broth (MHB, Merck, Darmstadt, Germany) was added to all wells of 96 microplates. Melittin and apamin (10 mg/mL) were added 50 µL each to the first well and serial dilutions were made in duplicate (500-0.98 µg/mL). Finally, 50 µL of bacterial suspensions at 0.5 McFarland turbidity were added to each of these wells. To control bacterial growth, only bacteria (positive control) was added to one last row of the microplate and only MHB (negative control) was added to one well. The microplate was incubated in an incubator at 37°C for 18-20 h. The lowest concentration at which no growth was observed the next day was considered as the MIC value. MICs of antimicrobials were also determined. After the

MICs were determined, 100 µL samples were taken from each well that did not show growth and dripped onto TSA. The prepared petri dishes were incubated in an incubator at 37°C for 18-20 h. MBCs were defined as the concentration at which no growth was observed [25].

- Determination of Time Dependent Kill Curves (Kinetic Kill Curves)

Time-dependent killing level was performed to show the duration of action of the agents used. Melittin and apamin concentrations were prepared at 2X MIC values for all bacteria. Eppendorfs were filled with 500 µL of 0.5 McFarland turbid bacterial suspension and 500 µL of each concentration of melittin and apamin components. Incubated at 37°C. At 0 s, 1 h, 2 h, 4 h, 6 h and 24 h, 50 µL from each tube was added to 450 physiological saline (0.9%) and spread inoculated onto 100 µL TSA medium. After 24 h of incubation at 37°C, colonies formed on the petri dishes were counted and the number of bacteria per mL (cfu/mL) was calculated by taking into account the dilution factor. The time and concentration dependent change of bactericidal effect was taken as log10 value and this change was analyzed. Time-death graph was drawn according to the values obtained, time was shown on the "x" axis and the logarithmic value of the number of bacteria was shown on the "y" axis [26].

- Antibiofilm

The antibiofilm ability of melittin and apamin on ESKAPE pathogens was tested using the microtiter plate method. The first step involved growing single colonies overnight in 5 mL Tryptic Soya Broth (TSB)containing 1% glucose at 37°C with 180 rpm shaking. 100 µL of the bacterial suspension adjusted to 0.5 McFarland was added to 900 µL of TSB containing 1% glucose. 200 µL of this suspension was added to the wells of 96 U-shaped microplates and simultaneously serially diluted melittin and apamin in the range of 500 to 0.98 µg/mL in a volume of 200 µL. Incubated at 37°C for 1 night with 180 rpm in a shaking incubator. Following the removal of the wells' contents, the wells were air dried and cleaned three times using 200 µL of phosphate buffer saline (PBS) regular saline. Following the addition of 200 µL of 95% methanol to each well, the wells were aspirated after 15 min, dried once more at room temperature, and then the dye was removed after 5 min of staining with 0.1% crystal violet in a 200 µL volume. Wells were washed 3 times with PBS and air-dried again. Finally, 200 µL of 33% glacial acetic acid for Gram-positive bacteria and 95% ethanol for Gram-negative bacteria were added to the wells and the absorbance at 600 nm was recorded using a microplate reader after 15 min at room temperature [27]. The minimum biofilm eradicating concentration (MBEK) was determined as the concentration at which the absorbance was less than or equal to the negative control. The percentage of biofilm inhibition was calculated as follows [28]: MBIC = [1-(OD test/OD control)] × 100.

Effects of Melittin and Apamin Components on The Proliferation of L929 Fibroblast Cells

- L929 Fibroblast Cell Culture

The effects of melittin and apamin on healthy cells were determined using mouse connective tissue fibroblast cells (L929). L929 fibroblast (CCL-1) cells were obtained commercially from ATCC (Manassas, USA). Before starting the cell culture study, medium containing DMEM (Dulbecco's Modified Eagle's Medium - high glucose ATCC, USA) + 10% fetal bovine serum (FBS; ATCC, USA) + penicillin/streptomycin (100 µg/mL; Gibco, USA) was prepared under sterile conditions in a UV cabinet.

Cells were suspended in medium and cultured in 25 cm^2 and 75 cm^2 cell culture flasks under aseptic conditions in an incubator at 37°C, 95% humidity and 5%CO_2. During the cell culture periods, the adhesion of the cells to the surface, their potential to fill the flask surface and the density of the cells were examined under an inverted microscope (ZEISS Primovert, GERMANY).

- Application of Melittin and Apamin *to L929 Fibroblast Cells and Calculation of IC50 Dose with Rreal-Time Cell Analysis System*

Melittin and apamin were mechanically prepared in cell culture medium. The determination of the number of cells to be used in the analysis to determine the IC50 dose of melittin and apamin to L929 fibroblast cells propagated by cell culture studies was performed using the real-time cell analysis system xCELLigence™ (ACEA Biosciences, Inc., San Diego, CA, USA). To determine the IC50 value, 50000 cells per well were seeded on xCELLigence E-platters and apamin and melittin were added to the medium at decreasing concentrations (5 µM, 4 µM, 3 µM, 2 µM, 1 µM, 500 nM and 250 nM) and measurements were taken at 15 min intervals for 120 h. For control, only medium was added. IC_{50} value was analyzed simultaneously according to logarithmic increase in the xCELLigence system [29].

Statistical Analysis

To evaluate the cell culture cytotoxicity results obtained from the system, statistical analyses were carried out using the xCELLigence system software. The system software fits an optional "sigmoidal dose–response equation" curve to the experimental data points in order to calculate logarithmic half maximum inhibitory concentration (log [IC_{50}) values. Statistical analyses were performed with Prism version 9 (GraphPad Software, California, USA). The t-test was used to determine the differences between more than one independent group. All

Table 1. *Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of melittin and apamin against ESKAPE bacteria*

Strains	MIC (µg/mL)		MBC (µg/mL)	
	Melittin	Apamin	Melittin	Apamin
E. faecalis ATCC 29212	500	500	500	500
S. aureus ATCC 29213	7.812	250	31.25	250
K. pneumoniae ATCC 700603	62.5	250	62.5	500
A. baumannii ATCC 19606	125	7.812	125	7.812
P. aeruginosa ATCC 27853	0.98	250	0.98	250
E. coli ATCC 25922	7.812	500	15.625	500

MIC: *minimum inhibitory concentration;* ***MBC:*** *minimum bactericidal concentration*

data are presented as mean ± SD. P<0.05 was considered statistically significant.

Results

Mass Analysis of Melittin and Apamin

LC-MS mass spectra were used to identify, characterize and quantify melittin and apamin compounds in bee venom, and these spectra are important for understanding their pharmacological effects and potential uses. The molecular mass of the apamin peptide (M): 2026.9037 Da, while the molecular mass of melittin peptide (M): 2845.7725 Da [21].

Antimicrobial Activities of Melittin and Apamin

- Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Values

The MIC and MBC activities of melittin and apamin on ESKAPE pathogens are given in *Table 1*. The MIC values of melittin on ESKAPE bacteria were between 500-0.98 µg/mL, while apamin was detected at concentrations of 500-7.812 µg/mL. MBC values were also similar to the MIC concentrations. The lowest MIC and MBC values were 0.98 µg/mL for melittin on *P. aeruginosa* ATCC 27853 and 7.812 µg/mL for apamin on *A. baumannii* ATCC 19606 strain *(Table 1).*

- Results for Time-Dependent Kill Curves

According to the analysis of time-dependent killing curves, melittin and apamin were found to be bactericidal against all ESKAPE pathogens. Melittin and apamin were 1st and 8th on *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, 1st and 12th on *K. pneumoniae* ATCC 700603, 12th and 8th on *A. baumannii* ATCC 19606, 2nd and 8th on *P. aeruginosa* ATCC 27853, and 12th and 1st on *E. coli* ATCC 25922, respectively *(Fig. 2).*

- Antibiofilm Activities of Melittin and Apamin

Biofilm inhibition activity of melittin and apamin was performed against ESKAPE isolates. As shown in *Table 2*, melittin and apamin inhibited biofilm for ESKAPE pathogens to varying degrees. The percentage of biofilm inhibition was higher for *E. faecalis, S. aureus, K. pneumoniae, A. baumannii* in the presence of melittin than apamin at the same concentrations. For *P. aeruginosa and E. coli,* the percentage of inhibition was higher in the presence of apamin. In all isolates, inhibition was observed in the presence of melittin and apamin at concentrations of 7.812 µg/mL and above. At concentrations of 3.9 µg/mL and below, melittin and apamin did not inhibit biofilm on *P. aeruginosa* (3.9 µg/mL), *K. pneumoniae* (1.95 µg/mL),

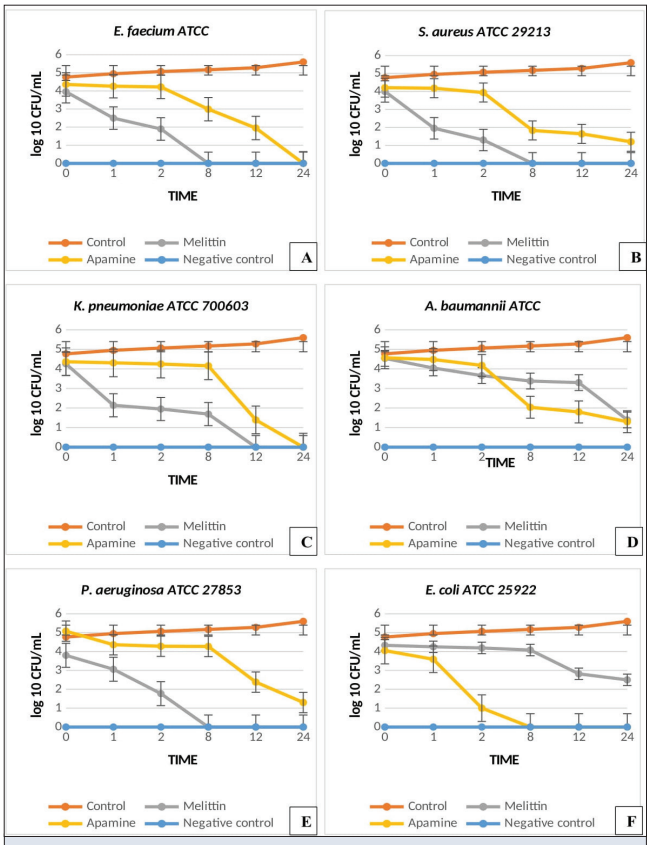

Fig 2. Time-kill assays of melittin and apamin for (A) *E. faecalis* ATCC 29212 (B) *S. aureus* ATCC 29213, (C) *K. pneumoniae* ATCC 700603, (D) *A. baumannii* ATCC 19606, (E) *P. aeruginosa* ATCC 27853, (F) *E. coli* ATCC 25922. X-axis represents killing time and Y-axis represents logarithmic (log) growth phase. Error bars indicate standard deviation

Table 2. *Percent inhibition (%) of biofilm formation of ESKAPE pathogens at different concentrations*

Microorganisms	Concentrations (µg/mL)																			
	500		250		125		62.5		31.25		15.625		7.812		3.9		1.95		0.98	
	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A
Enterococcus faecium	81.9	77.5	79.4	77.3	78.3	71.1	76.5	70.3	72.3	69.9	68.4	67.4	66.4	64.9	63.4	60.8	59.6	59.1	58.7	57.0
Staphylococcus areus	48.0	16.9	45.3	7.7	43.3	3.9	38.6	3.7	32.5	3.6	28.2	1.8	27.3	0.3	14.3	0	10.7	0	2.8	0
Klebsiella pneumoniae	59.6	51.9	49.5	48.4	41.7	37.7	32.2	37.1	18.9	35.6	16.7	33.2	8.4	32.3	4.8	31.0	0	30.5	0	8.8
Acinetobacter baumannii	59.6	53.3	59.5	40.4	51.4	37.0	48.1	30.5	40.6	21.4	40.0	19.1	43.5	21.5	32.1	10.0	25.8	7.6	27.5	5.2
Pseudomonas aeruginosa	57.6	62.7	53.7	60.4	53.3	57.6	43.1	58.2	39.9	57.6	28.6	56.4	8.5	53.9	0	53.0	0	52.9	0	50.0
Escherichia coli	62.0	68.9	56.8	48.5	54.1	46.7	46.6	46.3	43.3	46.0	42.0	46.4	41.8	45.3	33.8	44.0	25.1	43.6	11.8	38.3

M: melittin; A: apamin

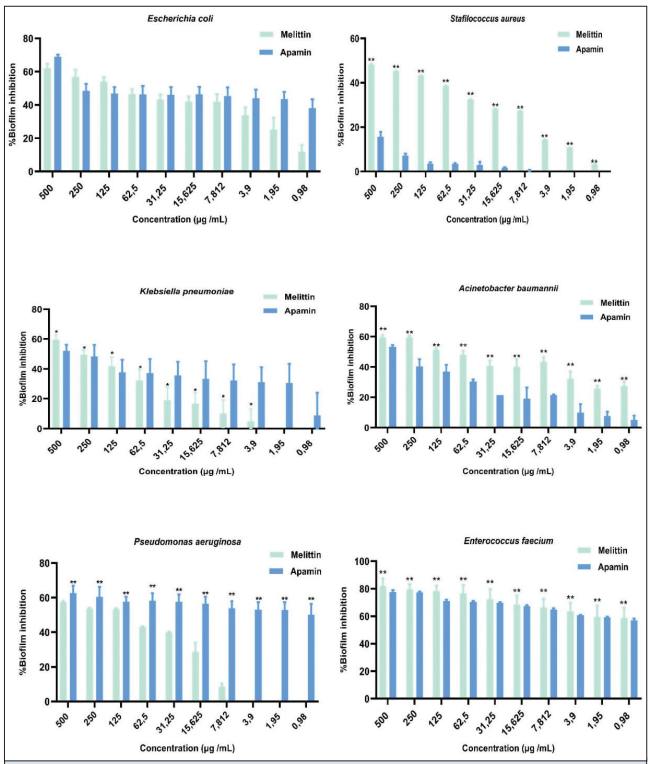

Fig 3. The graph shows the biofilm inhibition activity of melittin and apamin against ESKAPE pathogens. Error bars represent standard deviations. *P<0.05 and **P<0.001. Data were analyzed using Graphpad t-tests (non parametric test)

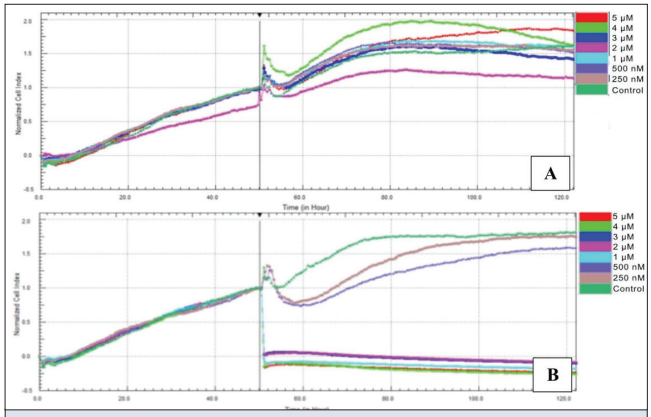

Fig 4. Time curves normalized cell index. Comparison of L929 cell proliferation after treatment with 5 µM, 4 µM, 3 µM, 2 µM, 1 µM, 500 nM and 250 nM (A): apamin and (B): mellitine. The arrow indicates the time of substance application

and *S. aureus* (3.9 µg/mL). Among the six pathogens, both melittin and apamin gave a high percentage of inhibition on *E. faecalis* isolate *(Fig. 3)*.

Effects of Melittin and Apamin Components on the Proliferation of L929 Fibroblast Cells

- Effects of Apamin on the Proliferation of L929 Cells

The software of the xCELLigence device automatically calculated IC_{50} values as 4.2 µM at 24 h, 4.9 µM at 48 h and 4.8 µM at 72 h after apamin treatment of L929 cells at different doses *(Fig. 4-A)*.

- Effects of Melittin on the Proliferation of L929 Cells

The software of the xCELLigence device automatically calculated IC_{50} values as 152 nM at 24 h, 87 nM at 48 h and 76 nM at 72 h after melittin treatment at different doses to L929 cells *(Fig. 4-B)*.

Discussion

The ability to effectively manage bacterial infections is threatened by rising rates of nosocomial infections and antibiotic resistance. Worldwide, the most common causes of nosocomial infections are ESKAPE pathogens, including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Escherichia coli*. The majority of the

ESKAPE pathogens are isolated from hospital units' blood, urine, wound swabs, respiratory samples, etc. (intensive care units (ICU), medical and surgical units) [31]. Even though hospitals are the main source, those with weakened immune systems-such as those with diabetes, chronic renal disease, lung disease, and cancer patients-are the most susceptible [32]. ESKAPE pathogens, which fall into many resistance categories (low-resistance, multi-drug and very drug resistant, pan-resistant bacteria), are the most common bacteria with an antibiotic resistance profile used as a sentinel in monitoring systems [33]. The multidrug resistance shown by these pathogens is a worldwide burden and treatment of patients with conventional antibiotics is becoming increasingly difficult or ineffective [34]. Antibiotics are known to interfere with the formation of cell walls, proteins, RNA, DNA, membrane stability, and energy metabolism. By altering these targets, deactivating antibiotics, or expelling them from cells, bacteria might develop resistance [34]. Combinations of antimicrobial drugs targeting different molecular targets than antibiotics can help combat mechanisms of resistance to antibiotics, broaden their antibacterial spectrum, reduce their toxicity and increase their efficacy [35]. It has been shown in various studies that melittin and bee venoms have broad-spectrum antibacterial effects and antibiofilm properties [36-41]. In this study, we tested the safety profile of melittin and apamin bee venoms from *Apis mellifera* L. using antimicrobial, antibiofilm and cytotoxicity methods.

Studies have shown the antibacterial activity of melittin [36-40]. Han et al.[38] in their antimicrobial analysis with melittin, reported the MIC value for *E. coli* as 0.125 µg/mL and for *S. aureus* as 0.06 µg/mL. Again, Maitip et al.[42] found that MIC and MBK values were 266 µg/mL for *E. coli*, respectively; 400 µg/mL; 400 µg/mL, 400 µg/mL for *K. pneumonia*; They found it to be 41 µg/mL and 41 µg/mL for *S. aureus*. In another experimental study investigating the antimicrobial activity of melittin, MIC and MBK values were given for *K. pneumoniae* (100 µg/mL; 300 µg/mL), *E. coli* (30 µg/mL; 60 µg/mL), *P. aeruginosa* (100 µg/mL;100 µg/mL), *A. baumannii* (30 µg/mL; 30 µg/mL) and *E. faecium* (1 µg/mL; 4 µg/mL) [43,44]. In our study, melittin showed antimicrobial properties at higher concentrations for *E. faecium* and at lower concentrations for other pathogens, giving different results from the literature. Apamin bee venom obtained from *Apis mellifera* L. showed antimicrobial activity on *E. coli* [45]. However, Kuzmenkov et al.[20] found that apamin did not show antimicrobial effect for *E. faecalis, S. aureus, E. coli* and *P. aeruginosa* up to a very high concentration of 50 µM. In our study, both melittin and apamin showed antimicrobial effect for all pathogens. Melittin showed antimicrobial effect at lower concentrations for all pathogens except *A. baumannii*. Apamin, on the contrary, showed antimicrobial effect at lower concentrations in *A. baumannii* pathogen compared to other pathogens.

Time-dependent killing assay was performed to monitor cell viability against time. Dosler et al.[46] determined the time-dependent killing assay results of melittin as 2-7 h for *P. aeruginosa*, *E. coli* and *K. pneumoniae*. In another study, time-dependent killing analysis results for *A. baumannii* (at 3rd, 5th, and 24th h), *S. aureus* (at 5th, and 24th h), and *K. pneumonia* (at 3rd, 5th, and 24th h) showed bactericidal effect at different concentrations [47]. The results of the time-dependent killing assay in our study showed that the two antimicrobial peptides, especially melittin, were rapidly bactericidal against *E. faecium, P. aeruginosa, K. pneumoniae* and *S. aureus* strains in a concentration-dependent manner within 1-8 h, followed by a significant logarithmic decrease at 1 h and bactericidal at 8 h. For *E. coli* and *A. baumannii*, a significant logarithmic decrease was observed at 12 and 24 h, which is consistent with the literature. In a study investigating the antibacterial effect of apamin on methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecalis*, carbapenem-resistant *E. coli*, carbapenem-resistant *K. pneumoniae* and carbapenem-resistant *Acinetobacter* species, it was found that the number of bacteria decreased by 4-6 logs within 1-24 h [48]. In our study, the times of logarithmic decrease and bactericidal properties were 8-24 h for *E. faecium, A. baumannii* and *S. aureus*, 12-24 h for *K. pneumoniae* and *P. aeruginosa* and 2-8 h for *E. coli*.

Medical science today faces the difficult problem of managing infections associated with biofilms. The antibiotic therapy used is typically insufficient to completely eradicate bacterial biofilms in this situation. This necessitates the use of large quantities of antimicrobial drugs, which are often equally ineffective, and sometimes even periodic prescriptions with a high risk of side effects and the emergence of new resistant strains [49]. It has been reported in different studies that melittin, an antimicrobial peptide, has strong antibacterial activity as well as strong antibiofilm activity [46,49-51]. Our study was consistent with the literature and showed a strong biofilm inhibition effect of melittin on ESKAPE pathogens with biofilm inhibition rates ranging from 82% to 8% in the concentration range of 500 to 7.812 µg/mL. To the best of our knowledge, there is no study investigating the antibiofilm activity of apamin. We determined the biofilm inhibition rate of apamin as 1.8% to 78% in the concentration range of 500-15.625 µg/mL.

While the antimicrobial effect of melittin and apamin is of importance, we investigated the survival rate of L929 fibroblasts. Zorilă et al.[52] reported a decrease of less than 50% in the viability of L929 cells up to 2.5 µM melittin and cell viability ranged from 55% to 63% for the highest

concentration tested. On the contrary, Klubthawee, et al.[53] reported that melittin was very cytotoxic against L929 cells and significantly reduced cell viability. In our study, it was found that the effective dose amount of melittin in L929 fibroblasts decreased with time. It was determined that melittin application above 500 nM had a lethal effect for L929 cells. In our study, similar to mellitine, apamin showed an effect at decreasing doses depending on time. However, apamin was not as effective as mellitine in L292 cells.

In conclusion, while antimicrobial resistance for ESKAPE pathogens is increasing and new antibiotics are not being developed to combat antibiotic-resistant microorganisms, melittin and apamin may be a promising alternative strategy against drug resistance of these microorganisms. In our study, we report the antimicrobial, antibiofilm and cytotoxic effects of melittin and apamin using ESKAPE ATCC strains. Melittin and apamin showed growth inhibition of ESKAPE microorganisms. Melittin and apamin also inhibited biofilm formation at extremely low concentrations. However, melittin and apamin synthesized in this study also had a positive effect on L929 cells. Our research thus indicates that melittin and apamin are potential antibacterial agents against strains of ESKAPE. To learn more about potential additional processes behind melittin and apamin's antibacterial action, more mechanistic research is being conducted. It is also necessary to look at the synergistic effects of melittin and apamin when used with antibiotics. The universality of the potential anticancer activity of these bee venoms also needs to be confirmed using cancer cell lines. This endemically synthesized melittin and apamin effective.

Declarations

Availability of Data and Materials: Materials and data sets from the study are available upon request from the corresponding author (E. Aydın).

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Author Contributions: All authors participated in designing the methodology, EA, MKG, AKS: writing the original manuscript, EA, SC, MKG, AKS: software and visualization, EA, SC, MKG, AKS: data curation, EA, SC, MKG, AKS: research and validation, EA, MKG, AKS: review and editing.

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Case Report

Surgical Correction of Congenital Meatal Stenosis Concurrent with Phimosis

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Abstract

An 18-month-old male cat presented with pollakiuria and hematuria. It had experienced misty urination and dampness around the genital area since the age of three months. Physical examination displayed small preputial and urethral openings, and subcutaneous cryptorchidism. Blood tests revealed elevated levels of blood urea nitrogen, creatinine, potassium, and phosphate. Radiography revealed a distended bladder. Castration and perineal urethrostomy were conducted to correct subcutaneous cryptorchidism and relieve urinary incontinence due to meatal stenosis and phimosis. Normal postoperative urination was observed; no complications were reported during the follow-up. This case highlights the successful surgical intervention of meatal stenosis concurrent with phimosis.

Keywords: Cat, Cryptorchidism, Perineal urethrostomy, Phimosis, Urethral stricture

Introduction

Feline urethral obstruction is a common disorder, with an incidence rate ranging from 1.5% to 9% [1]. The etiology of urethral obstruction is mostly acquired, including urethral plug, calculi, stricture, and neoplasia, although congenital anomalies have also been documented [2]. Meatal stenosis and phimosis, contributing to urethral obstruction, are often acquired through trauma and excessive grooming [3]. Clinical manifestations such as stranguria, pollakiuria, hematuria, and vomiting vary based on the extent of the obstruction. Diagnosis of meatal stenosis and phimosis involves visual examination of the penis and preputial ring. The management of both conditions typically requires surgical intervention via perineal urethrostomy (PU). Nonetheless, preputioplasty has been effective in treating phimosis, and wedge meatoplasty can be an option for addressing meatal stenosis [3-7].

This case report demonstrates the urinary obstruction resulting from suspected congenital meatal stenosis concurrent with phimosis in a cat. Both meatal stenosis and phimosis were successfully managed through PU, and the obstructive uropathy was resolved without complications.

Case History

Written informed consent was obtained from the owner for the participation of the animal in this study.

An 18-month-old intact male domestic short-haired cat weighing 3.5 kg presented with pollakiuria and hematuria. The cat had been experiencing sporadic voiding difficulties since the age of three months, characterized by a misty pattern during urination and dampness around the genital area. There was no evidence of trauma or excessive licking of the genital region. While the vital signs were normal during the physical examination, abnormal findings included a small preputial opening, a small urethral opening, and a misty pattern during urination. The prepuce could not be fully retracted, exposing only the tip of the penis owing to its small opening. Attempts to assess patency using a 3-fr tomcat urinary catheter were unsuccessful because of the narrow urethral opening. Subcutaneous cryptorchidism was observed in the left inguinal region.

Blood tests, including complete blood count, blood gas analysis and serum blood chemistry, revealed increased blood urea nitrogen (BUN, 182.1 mg/dL [reference interval {RI} 15-37 mg/dL]), increased creatinine (13.2 mg/dL [RI 0.7-2.1 mg/dL]), hyperkalemia (8.1 mmol/L [RI 3.2-5.5 mmol/L]) and hyperphosphatemia (9.4 mg/dL [RI 2.6-6.4 mg/dL]). Radiography revealed no abnormalities, except for a distended bladder *(Fig. 1)*. Cystocentesis was performed to relieve urinary retention temporarily.

The patient underwent castration and PU to address the voiding difficulties and correct cryptorchidism. Intravenous propofol (5 mg/kg, Provive, Myungmoon Pharm. Co., Ltd.; Seoul, South Korea) was administered to induce anesthesia. Under general anesthesia using 2.0% isoflurane (Terrell; Piramal Critical Care; Bethlehem, PA, USA) with 100% oxygen ventilation and an intravenous infusion of 0.9% NaCl solution (2 mL/kg/h; 0.9% NS, JW Pharm. Co., Ltd.; Gwacheon, South Korea), the patient was maintained in the perineal position throughout the surgery. Conventional castration was performed prior to PU. While the right testicle was in a normal position, the left testicle was located in the subcutaneous inguinal region. The removed testicle showed hypoplasia on the left side (10.5 x 5.5 mm) compared to the right testicle (15.8 x 12.3 mm) *(Fig. 2)*. A partial prepuce incision exposed the penis. An incision at the penile tip revealed a urethral stricture, obstructing the insertion of a urinary catheter despite the extension of the incision *(Fig. 3-A)*. Subsequently, PU was conducted. When the perineal urethra was exposed, a urinary catheter was inserted retrogradely to evaluate patency, revealing only urethral strictures in the glans penis region. After PU, urethral patency assessed by insertion of a urinary catheter, indicating no evidence of constriction in the urethral passage *(Fig. 3-B)*.

The surgery was uneventful, and remifentanil (2.5 µg/kg/h; Tivare, BCWORLD Pharm. Co., Ltd.; Yeojoo, South Korea) was administered intravenously for pain management during and after surgery. Normal urination was observed during hospitalization. The patient was discharged 3 days after surgery and received amoxicillin/clavulanate (13.75 mg/kg; AMOCLA, KUHNIL Pharm. Co., Ltd.; Cheonan, South Korea), metronidazole (10 mg/kg; Flasinyl, HK inno.N; Cheongju, South Korea), and famotidine (1 mg/kg; Famotidine, NELSON; Cheongju, South Korea) orally for 7 days. Post-surgical assessment was conducted 1 week, 3 weeks, 6 weeks, and 3 months after the surgery. The blood analysis conducted 3 weeks after the operation showed marked improvement in BUN (21 mg/dL [RI 15-37 mg/dL]), creatinine (1.1 mg/dL [RI 0.7-2.1 mg/dL]), potassium (4.7 mmol/L [RI 3.2-5.5 mmol/L]), and phosphate (5.8 mg/dL [RI 2.6-6.4 mg/dL]). The owner did not observe any urinary dysfunction

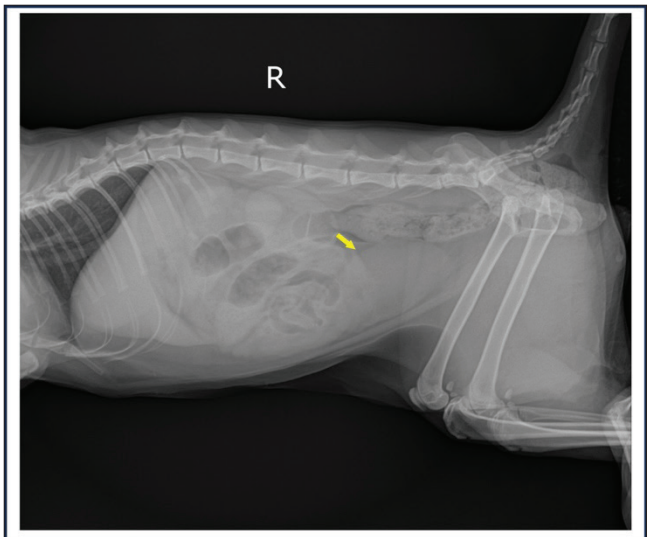

Fig 1. Preoperative abdominal radiography revealed urinary bladder distention *(arrow)*. No other factors contributing to urinary blockage were detected

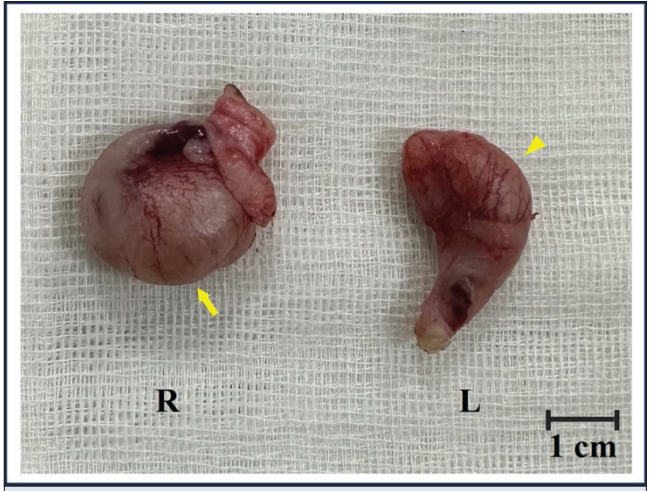

Fig 2. Gross pathology of the testicles showed the left testicle (10.5 x 5.5 mm) *(arrowhead)* is smaller than the right testicle (15.8 x 12.3 mm) *(arrow)* indicating left testicular hypoplasia

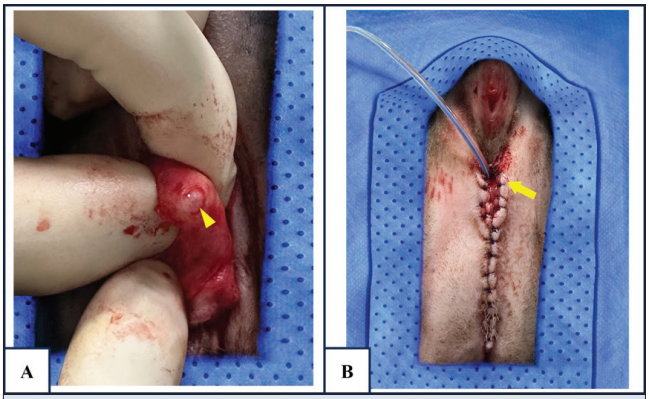

Fig 3. Urethral patency of the patient. **A-** The penis was exposed by a partial preputial incision, and an incision at the penile tip revealed the presence of meatal stenosis *(arrowhead)*, **B-** Urethral patency was assessed through the normograde insertion of a urinary catheter *(arrow)*, demonstrating the absence of stricture in the urethra

or surgical site problems, and no complications were reported during the three-month follow-up period.

Discussion

This case report documents the successful surgical management of urethral obstruction resulting from congenital meatal stenosis along with phimosis in a cat.

When initially presented to the hospital, the patient was suspected with urinary obstruction due to clinical signs, elevated BUN and creatinine levels, and electrolyte imbalance, necessitating immediate treatment [8]. Despite considering urethral catheterization as the primary method to alleviate the obstruction, the narrow preputial and urethral openings of the patient prevented the insertion of a 3-fr tomcat catheter. Visual inspection and radiography revealed no evidence of other causes of urinary obstruction. Although cystocentesis provided temporary relief for the distended bladder, structural urethral obstruction required surgical intervention.

In this case, PU was chosen as the surgical technique to address both meatal stenosis and phimosis. The surgical approach for phimosis varies based on the presence of penile-preputial adhesions [4]. These methods include circumferential preputioplasty, preputial urethrostomy, and PU [6,7]. Feline meatal stenosis requires PU or wedge meatoplasty [5,9]. Due to the potential risk of urethral stricture associated with both circumferential preputioplasty and wedge meatoplasty, the concomitant application of these surgical techniques was not conducted [10,11]. The wedge meatoplasty or amputation at the penile tip was considered incompletely resolving the urethral stricture at the distal urethra, necessitating PU to alleviate urinary obstruction. During surgery, a catheter was used to verify urethral patency and uncover a stricture solely in the penile urethra. Consequently, PU emerged as the optimal decision to address the structural obstruction arising from both phimosis and meatal stenosis.

The evaluation of PU outcomes focused on identifying complications, with short- and long-term issues reported in 62.2% and 14.3% of cases, respectively [12]. Possible short- and long-term complications include sterile cystitis, repeated urethral obstruction, urinary tract infection, urinary incontinence, urethrostomy stricture, and urine leakage into the subcutaneous space of the peristoma. In this case, no complications related to urinary clinical signs were identified for three-months follow-up period.

Disorders of sexual development (DSDs) are abnormalities resulting from inadequate development of the reproductive system. DSDs in tomcats are infrequently reported and manifest as ambiguous genitalia, penile frenulum, penile hypoplasia, cryptorchidism, hypospadias, and phimosis. DSDs often occur alongside other congenital abnormalities in cats. Cryptorchidism is associated with hypospadias, and penile, preputial, and testicular hypoplasia [1]. Phimosis is also frequently observed in conjunction with penile hypoplasia [4]. In this case, the patient experienced urinary obstruction due to congenital phimosis concomitant with meatal stenosis. While feline meatal stenosis is typically considered an acquired abnormality rather than a congenital defect, the patient in this case exhibited obstructive clinical signs shortly after birth with no evidence of traumatic events causing the meatal stenosis [3]. In addition, subcutaneous cryptorchidism was identified, along with testicular hypoplasia. Thus, the patient exhibited a combination of DSDs, making this case distinct and offering valuable insights into feline DSDs.

One limitation of this report was the absence of positive cystourethrography results to identify and assess the location of the urethral stricture. Positive cystourethrography is less sensitive for detecting urethral strictures at the penile tip, and the diagnosis of meatal stenosis relies on direct catheterization [3]. Urethral patency was examined through direct insertion of a catheter during the operation, leading to the diagnosis of meatal stenosis.

This case report represents the documentation of congenital phimosis concurrent with meatal stenosis in a cat. The patient also presented with cryptorchidism and testicular hypoplasia, and simultaneous occurrence of DSDs is rarely observed in cats. This case report highlights the clinical insight that male kittens displaying urethral obstruction and presenting with a misty urinary pattern should be suspected of having congenital meatal stenosis and phimosis. In cases of DSDs, a thorough examination of other DSDs should be conducted. Moreover, it is advisable to contemplate PU as a surgical technique to alleviate urinary blockage caused by the simultaneous presence of phimosis and meatal stenosis.

Declarations

Availability of Data and Materials: The datasets analyzed during the study are available from the corresponding author (S. LEE) on request.

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Competing Interests: The authors declared that there is no conflict of interest.

Authors' Contributions: Conceptualization was done by SK and

SL, data curation was done by SK and YK, visualization was done by SK and YK, and the article was written by SK, YK and SL.

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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.

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Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

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7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

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References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

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DOI number should be added to the end of the reference.

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