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E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 5, Year: 2024

THE REFEREES LIST OF THIS ISSUE *(in alphabetical order)*

Abdullah DİKİCİ	Uşak Üniversitesi Mühendislik ve Doğa Bilimleri Fakültesi
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E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 5, Year: 2024

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 5, Year: 2024

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
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Review Article

Review of Toxic Trace Elements Contamination in Some Animal Food Products in Different Countries

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Abstract

Meat, chicken, eggs and milk are all important foods worldwide because of their energetic and nutritious constituents beneficial to human health. This review aims to analyze the results of the studies carried out on the contamination of these foods (meat, chicken, eggs and milk) by the toxic trace elements As, Cd and Pb in different countries, and to compare their values with the international regulatory limits. According to the data of the various studies analyzed, all the studied matrices have been contaminated with these toxic metals. The concentrations reported differ for each matrix according to their analyzed tissues, their geographical location, their age and the food types chosen. The concentrations involved also are highly dependent on the studied trace element. Most of the reported concentrations in these foods exceed the international regulatory thresholds. Therefore, it is important to perform regular monitoring studies for all these foodstuffs along with corresponding health risk assessment estimates as well as carrying out studies to better identify the origin of the high levels of these contaminants and seek solutions to prevent major human poisonings and to ensure the safety of these foods.

Keywords: Environmental contaminants, Food, Heavy metals, Health risk assessment, Regulatory thresholds

Introduction

Food such as meat, poultry, eggs and milk are a fundamental source of high quality animal proteins with bioactive peptides, lipids, minerals and vitamins (vitamins B3, B6, B12, and D) which provide adequate energy for daily needs and are essential for the growth and well-being of human beings [1-4]. But these foods can be contaminated by several environmental contaminants such as toxic trace elements [1]. Trace elements are "metallic and non-metallic" chemical elements that are found in the environment in small quantities (less than 100 mg/kg) and are found in different chemical forms, they have a higher density than water [5,6]. Among these trace elements, some are essential and are required by the organism in small quantities to ensure its proper physiological functioning such as copper (Cu), nickel (Ni), zinc (Zn), iron(Fe) and manganese (Mn) [7-9]. In contrast to these essential trace elements, the toxic trace elements have no physiological role and are classified in the list of the most toxic substances known, i.e. arsenic (As), cadmium (Cd) and lead (Pb) [1,2,7,8]. These latter

elements are a major health and food safety concern which pose a large threat to the environment and human health due to their toxicity, persistence and non-biodegradability in the environment as well as bioaccumulation in the food chain [10-13]. Trace element pollution can originate from natural sources such as soil erosion and volcanoes and from anthropogenic activities including mining, smelting, wastewater disposal, and industrial discharges *(Fig. 1)*. The means of transport that release the trace elements, and the application of pesticides and fertilizers are the major sources of exposition [2,6,7]. Once in the environment, they can readily be introduced into plants, animal tissue and humans *(Fig. 1)* [2]. Dietary intake is considered the primary route of human exposure to toxic trace elements (50%), compared to other exposure routes [2]. These toxic trace elements in the body can lead to undesirable chronic effects; e.g. arsenic typically causes nervous, gastro-intestinal, cardiovascular, renal, pulmonary, reproductive disorders, and keratosis, melanosis and other skin disorders as well as cancers of various organs (liver, kidney and intestines) [14-17]. Exposure to cadmium causes kidney, liver, lung, and neurological and bone damage (itai - itai diseases) [18,19]. Cd causes damage to the placenta which leads to infertility, congenital malformations and abortion; in males, the damage caused by Cd has resulted in decreased sperm motility, testicular deformation and prostate cancer [19]. Exposure to Pb can cause neurological disorders that are expressed by symptoms such as learning and pronunciation problems, memory loss, depression and general fatigue [12,20,21]. It also causes other problems such as gastrointestinal disorders and anemia [21]. Thus, this review aims to analyze and evaluate the results obtained in different studies on the levels of trace element contamination of meat, chicken, eggs and milk by these three toxic elements As, Cd and Pb, and to compare concentrations with the regulatory limits to assess if they constitute a health risk for the consumer. The articles used in this study to analyze and evaluate previous studies are 55 research articles representing 10% of total papers collected from four main datavases which are Google Scholar, SNDL (National System of Online Documentation), PubMed, and ResearchGate. The 55 research articles were chosen since they precisely met our criterion of the analysis of critical toxic trace elements in foods from animal origin that we intended to examine. The period of download was from April 2023 to November 2023. These collected and analyzed articles come from different continents: Asia, Africa, Europe and America, with those from Asia being the most numerous.

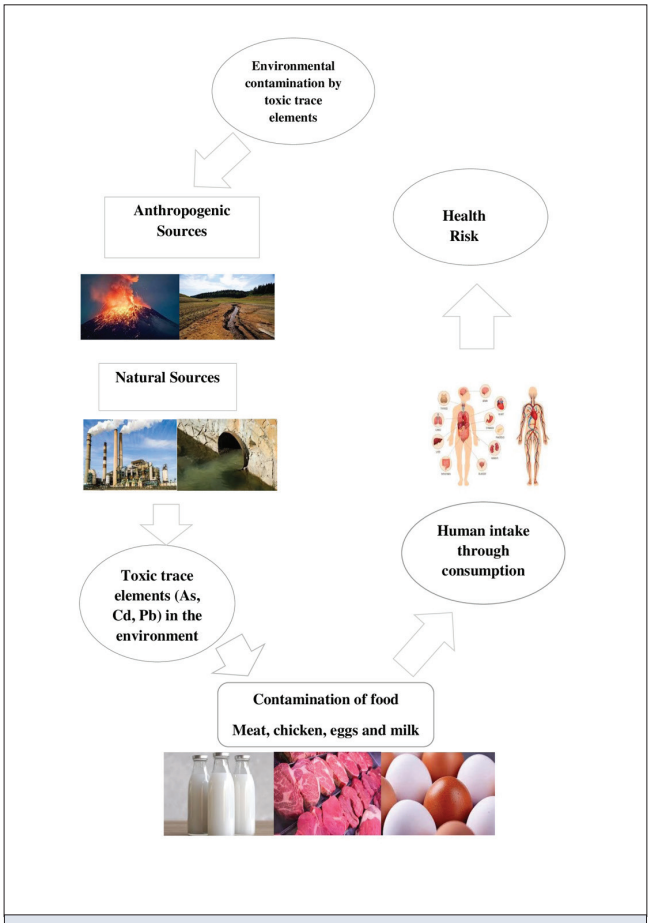

Fig 1. Sources of Toxic trace elements in the environment and contamination of food and their humain impact

Toxic Trace Elements in Food

Concentration of As, Cd and Pb in Different Tissues of Chicken

The results of the literature *(Table 1)* on the concentrations of As, Cd and Pb are very different in each tissue analyzed, in each species of chicken studied and also in each region of origin. For As, the highest concentration was reported by Bazzaz et al.[22] in the thigh and the breast meat of cock from Brazil (1.82 and 1.82 mg/kg w.w., respectively) and Türkiye (1.57 and 1.77 mg/kg w.w., respectively), respectively. The same authors showed that breast meat accumulates As more than thigh meat, which would support the observations that As concentrations vary according to muscle type for the same animal species Mottalib et al. [23]. reported that the highest concentrations of As in liver (0.94 mg/kg w.w.) in the broiler chicken from Bangladesh and the concentrations were not the same in the three species studied. The lowest concentration of As (0.06 mg/ kg w.w.) was measured by Uluozlu et al.[24] in kidney and liver of the cock chicken from Türkiye. These maximum concentrations in the liver of the majority of the results compared to other tissues could be related to the important role liver plays in trace element metabolism. In the case of Cd, Kamaly and Sharkawy [25] registered the highest concentrations in the meat of six brands of chicken (14; 13.01; 10.93; 10.15; 9.63; 5.87 mg/kg w.w.) which were higher than those in the liver (0.01; 0.02; 0.03; 0.05

Table 1. *Summary of the concentrations of trace elements in different parts of chicken*

Trace Elements	Type of Chicken (local names)	Tissues	Concentration (mg/kg wet weight)	Countries	References
As	Cock	Heart	0.06	Türkiye	Uluozlu et al.[24]
		Kidney	0.09		
		Liver	0.06		
		Meat	0.07		
		Breast	0.45	Bangladesh	Mottalib et al.[23]
		Liver	0.37		
	Broiler	Breast	0.63		
		Liver	0.94		
	Layer	Breast	0.21		
		Liver	0.24		
	Broiler	Meat	0.04	Bangladesh	Ullah et al.[28]
	Local		0.04		
	Sonali		0.04		
	Cock	Meat	0.19	Malaysia	Abduljaleel et al.[27]
		Liver	0.51		
		Tight	1.57	Türkiye	Bazzaz et al.[22]
		Breast	1.77		
		Tight Breast	1.82 1.82	Brazil	
		Meat	0.43	Bangladesh	Ahmed et al.[29]
		Meat	0.02	Brazil	Ng et al.[30]
		Meat	0.31	India	Das et al.[31]
		Meat	0.43	Bangladesh	Shaheen et al.[32]
Cd	Cock	Heart	0.25	Türkiye	Uluozlu et al.[24]
		Kidney	0.25		
		Liver	2.24		
		Meat	6.09		
		Heart	0.70	Türkiye	Yilmaz and Gecgel [33]
		Liver	0.05		
		Meat	0.01		
	Broiler	Meat	0.01	Bangladesh	Ullah et al.[28]
	Local		0.01		
	Sonali		0.01		
	Cock	Meat	0.23	Bangladesh	Shaheen et al.[32]
		Meat	0.15	Malaysia	Abduljaleel et al.[27]
		Liver	0.15		
	Brands 1 of poultry	Meat	5.87	Egypt	Kamaly and Shakawy [25]
		Liver	0.05		
	Brands 2 of poultry	Meat	0.63		
		Liver	0.01		
	Brands 3 of poultry	Meat	13.01		
		Liver	0.02		
	Brands 4 of poultry	Meat	14.00		
		Liver	10.93		
	Brands 5 of poultry	Meat	0.03		
		Liver	0.02		
	Brands 6 of poultry	Meat	10.15		
		Liver	0.03		
	White cornish	Meat	0.04	Egypt	Elsharawy [34]
	Layer	Liver	0.06	Egypt	El Bayomi et al.[26]
		Meat	0.06		
	Broiler	Liver	0.10		
		Meat	0.09		

Table 1. *Summary of the concentrations of trace elements in different parts of chicken (continued)*

Trace Elements	Type of Chicken (local names)	Tissues	Concentration (mg/kg wet weight)	Countries	References
Pb	Cock	Heart	0.04	Türkiye	Uluozlu et al.[24]
		Kidney	0.02		
		Liver	0.12		
		Meat	0.40		
		Heart	0.01	Türkiye	Yilmaz and Gecgel [33]
		Liver	0.03		
		Meat	0.02		
	Broiler	Meat	0.59	Bangladesh	Ullah et al.[28]
	Local		0.64		
	Sonali		1.02		
	Cock	Meat	0.37	Bangladesh	Shaheen et al.[32]
	Brand 1 of poultry	Meat	0.10	Egypt	Kamaly and Sharkawy [25]
		Liver	2.57		
	Brands 2 of poultry	Meat	0.03		
		Liver	3.15		
	Brands 3 of poultry	Meat	0.04		
		Liver	2.56		
	Brands 4 of poultry	Meat	0.04		
		Liver	2.58		
	Brands 5 of poultry	Meat	0.02		
		Liver	0.03		
	Brands 6 of poultry	Meat	0.30		
		Liver	5.55		
	White cornish	Meat	0.30	Egypt	Elsharawy [34]
	Cock	Tight	0.43	Türkiye	Bazzaz et al.[22]
		Breast	0.49		
		Tight	0.55	Brazil	
		Breast	0.50		
		Meat	0.21	Malaysia	Abduljaleel et al.[27]
		Liver	0.35		
	Layer	Liver	0.21	Egypt	El Bayomi et al.[26]
		Meat	0.10		
	Broiler	Liver	0.34		
		Meat	0.31		

As: Arsenic, Cd: Cadmium, Pb: Lead

mg/kg w.w.). Uluozlu et al.[24] also recorded the highest concentration of Cd in the meat of cock chicken from Türkiye (6.09 mg/kg w.w.). This could be a function of the strong assimilation of this metal in the muscle compared to that in the other organs (liver, kidney heart).

Regarding the levels of Pb, the authors reported different concentrations depending on the tissues and chicken species studied as well as the area of origin *(Table 1)*. Kamaly and Sharkawy[25] measured the highest concentration of Pb in the liver of the six brands of cock from Egypt. The liver accumulates more Pb than other tissues in cock [25-27]. This is linked to the role of the liveras an organ of metabolism, which is why it is the most important target for this metal.

Concentrations of As, Cd and Pb in Meat of Cattle and Sheep

The studies examined have found different concentrations of As in cattle meat *(Table 2)*, the highest of which (5.6 and 3.7 mg/kg w.w.) were recorded by Sathyamoorthy et al.[10] in India. Adjei et al.[35] registered a differences in values from four study sites where the highest was 0.28 mg/kg w.w. and the lowest 0.00 mg/ kg w.w. which was the lowest concentration recorded compared to the others results. For As in sheep, the highest concentration (0.34 mg/kg w.w.) was measured by Abd-Elghany et al.[36] in Kuwait and the lowest concentrations (0.00 mg/kg w.w.) were registered by Xiang et al.[37] from China. Regarding Cd concentrations in meat of cattle, Sathyamoorthy et al.[10] in India reported the highest concentrations (6.6 and 5.1 mg/kg w.w.) whereas the lowest concentration was recorded by Di Bella et al.[38] in Italy where the value was below the limit of detection (*ND*) *(Table 2)*. The highest concentration of Cd in the meat of sheep (0.23 mg/kg w.w.) was reported by Raeeszadeh et al.[39] in Iran and the lowest concentrations (0.00 mg/ kg w.w.) was obtained

Table 2. *Summary of the concentrations of trace elements in meat of cattle and sheep*

Trace Elements	Type of Meat	Concentrations (mg/kg wet weight)		Countries		References
As	Cattle	0.01		Italy		Di Bella et al.[38]
		2.73		Bangladesh		Chowdhury et al.[41]
		0.00		Ghana		Nkansah and Ansah [43]
		3.7		Site 1	India	Sathyamoorthy et al.[10]
		5.6		Site 2		
		0.28		Site 1	Ghana	Adjei et al.[35]
		0.00		Site 2		
		0.00		Site 3		
		0.01		Site 4		
		0.57		Bangladesh		Shaheen et al.[32]
	Sheep	0.23		Iran		Raeeszadeh et al.[39]
		0.01		Ghana		Nkansah and Ansah [43]
		0.34		Kuwait		Abd-Elghany et al.[36]
		Shoulder clod	0.00	China		Xiang et al.[37]
		Tenderloin	0.00			
		Neck	0.00			
		Rump	0.00			
		Mutton tripe	0.00			
		Intercostal meat	0.00			
		0.14		Bangladesh		Shaheen et al.[32]
Cd	Cattle	ND		Italy		Di Bella et al.[38]
		0.02		Thailand		Jankeaw et al.[44]
		0.00		Ghana		Adzitey et al.[45]
		0.01		Bangladesh		Kamal et al.[46]
		0.4		Uganda		Kasozi et al.[40]
		0.00		Bangladesh		Chowdhury et al.[41]
		0.07		Ghana		Nkansah and Ansah [43]
		0.00		Nigeria		Sabuwa and Nafarnda [48]
		6.6		Site 1	India	Sathyamoorthy et al.[10]
		5.1		Site 2		
		0.10		Site 1		Adjei et al.[35]
		0.05		Site 2		
		0.04		Site 3		
		0.00		Site 4		
		0.12		Bangladesh		Shaheen et al.[32]
	Sheep	Young	0.03	Egypt		Darwish et al.[42]
		Aged	0.06			
		0.23		Iran		Raeeszadeh et al.[39]
		0.01		Ghana		Nkansah and Ansah [43]
		0.30		Kuwait		Abd-Elghany et al.[36]
		0.14		Bangladesh		Shaheen et al.[32]
		0.01		Egypt		Abou-Arab [47]
		Shoulder clod	0.00	China		Xiang et al.[37]
		Tenderloin	0.00			
		Neck	0.00			
		Rump	0.00			
		Mutton tripe	0.00			
		Intercostal meat	0.00			

Table 2. *Summary of the concentrations of trace elements in meat of cattle and sheep (continued)*

Trace Elements	Type of Meat	Concentrations (mg/kg wet weight)		Countries		References
Pb	Cattle	0.01		Italy		Di Bella et al.[38]
		0.04		Thailand		Jankeaw et al.[44]
		0.00		Ghana		Adzitey et al.[45]
		0.09		Bangladesh		Kamal et al.[46]
		5.4		Uganda		Kasozi et al. [40]
		4.62		Bangladesh		Chowdhury et al.[41]
		1.15		Ghana		Nkansah and Ansah [43]
		0.05		Nigeria		Sabuwa and Nafarnda [48]
		4.6		Site 1	India	Sathyamoorthy et al.[10]
		2.4		Site 2		
		0.09		Site 1	Ghana	Adjei et al.[35]
		0.01		Site 2		
		0.04		Site 3		
		0.00		Site 4		
		0.48		Bangladesh		Shaheen et al.[32]
	Sheep	Young	0.07	Egypt		Darwish et al.[42]
		Aged	0.25			
		Shoulder clod	0.00	China		Xiang et al.[37]
		Tenderloin	0.00			
		Neck	0.00			
		Rump	0.00			
		Mutton tripe	0.00			
		Intercostal meat	0.00			
		0.37		Ghana		Nkansah and Ansah [43]
		11.79		Iran		Raeeszadeh et al.[39]
		0.48		Kuwait		Abd-Elghany et al.[36]
		0.01		Egypt		Abou-Arab [47]
		0.15		Bangladesh		Shaheen et al.[32]

As: Arsenic, Cd: Cadmium, Pb: Lead, ND: Not Detected

in different areas of China by Xiang et al.[37]. For Pb in the meat of cattle, Kasozi et al.[40] measured the highest concentration (5.4 mg/kg w.w.) in meat from Uganda. Chowdhury et al.[41] in Bangladesh and Sathyamoorthy et al.[10] in India also recorded high concentrations of Pb with a similar value (4.6 mg/kg w.w.). The concentrations recorded in the other studies were negligible *(Table 2)*. For Pb in sheep meat from Iran, Raeeszadeh et al.[39] measured a significantly high concentration (11.79 mg/kg w.w.) which was higher compared to the results of all the other studies and the lowest concentrations were recorded by Xiang et al.[37] from different locations in China. Darwish et al.[42] showed that there is a difference in Pb concentration in sheep meat as a function of the animals' age, the concentration in meat of older sheep (0.25 mg/kg w.w.) was higher than that of younger individuals (0.07 mg/kg w.w.). The results of these different studies *(Table 2)* show that there are important variations in concentrations of the accumulation of trace elements (i.e. As, Cd and Pb) in cattle and sheep meat underscoring the importance of such regional studies on the contents of these elements in comestible species.

Concentrations of As, Cd and Pb in Eggs

The concentrations of the targeted trace elements (As, Cd and Pb) in eggs *(Table 3)* are quite different. For the same metal, the analyticle results differ depending on the type of egg and for the same type of egg the results are different depending on the method of breeding of the species producing these eggs and therefore on its food type and the animals' geographic location. For As, the higest concentrations were reported by Shaheen et al.[32] from Bangladesh in chicken and duck eggs (0.30 and 034 mg/kg w.w., respectively). Nisianakis et al.[49] in Greece found similar concentrations (0.01 mg/kg w.w.) in three types of eggs (chicken, duck and goose), a finding that is explained by the fact that these species of hens have the same rearing method and especially the same diet. These values were higher than those reported by the same

Table 3. *Summary of the concentrations of trace elements in eggs of different poultry species*

Trace Elements	Type of Eggs	Concentrations (mg/kg wet weight)		Countries		References
As	Chicken	0.01		Greece		Nisianakis et al.[49]
	Türkiye	0.00				
	Duck	0.01				
	Goose	0.01				
	Chicken	Balady eggs	0.14	Egypt		Saad Eldin and Raslan [50]
		Commerciel eggs	0.12			
		Organic eggs	0.04			
		Local 1	BDL	Bangladesh		Samad et al.[51]
		Local 2	BDL			
		Local 3	BDL			
		Local 4	BDL			
		Local 5	BDL			
		Local 6	BDL			
		0.30		Bangladesh		Shaheen et al.[32]
	Duck	0.34				
	Chicken	0.02		North	Egypt	Hashish et al.[56]
		0.03		Middle		
		0.02		South		
		0.02		Upper Egypt		
		0.00		France		Leblanc et al.[54]
		0.02		Egypt		Ferweez et al.[57]
		0.00		United Kingdom		Ysart et al.[55]
Cd	Chicken	0.00		Greece		Nisianakis et al.[49]
	Türkiye	0.00				
	Duck	0.00				
	Goose	0.00				
	Chicken	Balady eggs	0.18	Egypt		Saad Eldin and Raslan [50]
		Commerciel eggs	0.09			
		Organic eggs	0.04			
		0.30		Bangladesh		Shaheen et al.[32]
	Duck	0.34				
	Chicken	0.18		Iran		Sobhan Ardakani et al.[53]
		Local 1	< 0.001	Bangladesh		Samad et al.[51]
		Local 2	< 0.001			
		Local 3	< 0.001			
		Local 4	< 0.001			
		Local 5	< 0.001			
		Local 6	< 0.001			
		0.01		North	Egypt	Hashish et al.[56]
		0.01		Middle		
		0.00		South		
		0.00		Upper Egypt		
		0.00		France		Leblac et al.[54]
	Duck	0.93		Indonesia		Asnawi [52]
	Chicken	0.03		Egypt		Ferweez et al.[57]
		0.00		United Kingdom		Ysart et al.[55]

Table 3. *Summary of the concentrations of trace elements in eggs of different poultry species (continued)*

Trace Elements	Type of Eggs	Concentrations (mg/kg wet weight)		Countries		References
Pb	Chicken	Balady eggs	0.34	Egypt		Saad Eldin and Raslan [50]
		Commercial eggs	0.18			
		Organic eggs	0.08			
		0.28		Bangladesh		Shaheen et al.[32]
	Duck	0.32				
	Chicken	0.29		Iran		Sobhan Ardakani [53]
		0.09		Egypt		Ferweez et al.[57]
		0.22		North	Egypt	Hashish et al.[56]
		0.30		Middle		
		0.17		South		
		0.17		Upper Egypt		
		0.01		France		Leblanc et al.[54]
		Local 1	0.37	Bangladesh		Samad et al.[51]
		Local 2	0.29			
		Local 3	0.51			
		Local 4	1.90			
		Local 5	0.50			
		Local 6	1.58			
		0.00		United Kingdom		Ysart et al.[55]

As: Arsenic, Cd: Cadmium, Pb: Lead, BDL: Below detection limit

authors for Türkiye. eggs (0.009 mg/kg w.w.). In Egypt, Saad Eldin and Raslan [50] reported high and almost similar concentrations for Ballady and comercial eggs (0.12 and 0.14 mg/kg w.w., respectively) the concentrations for which are much higher than that obtained for the organic eggs (0.04 mg/kg w.w.). Samad et al.[51] from Bangladesh reported the negligible and lowest recorded values of As in chicken eggs which were below the limit of detection (BDL).

For Cd, Asnawi [52] in Indonesia reported the highest value (0.93 mg/kg w.w.) in duck eggs followed by the results registered by Shaheen et al.[32] in Bangladesh in chicken and duck eggs (0.30 and 0.34 mg/kg w.w., respectively). Sobhan Ardakani et al.[53] in Iran also registered the highest concentration in chicken eggs (0.18 mg/kg w.w.). Nisianakis et al.[49] in Egypt, Leblanc et al.[54] in France, Ysart et al.[55] in United Kingdom, Hashish et al.[56] in upper and south Egypt and Samad et al.[51] in Bangladesh reported the lowest concentrations in chicken eggs. The highest concentrations in chicken eggs were reported by Shaheen et al.[32] in Bangladesh which recorded similar values in chicken and duck eggs. Hashish et al.[56] in Egypt reported different concentrations in chicken eggs from different regions for which the highest levels were found in the eggs from the north and middle regions of the country, and the lowest values were registered in those from the south and in Upper Egypt.

For Pb, the highest concentrations were registered in chicken eggs by Samad et al.[51] (1.9; 1.58; 0.51 mg/kg w.w.) from different sites in Bangladesh, followed by the results obtained by Hashish et al.[56] from Egypt (0.34 and 0.30 mg/kg w.w., respectively) in chicken eggs. Shaheen et al.[32] in Bangladesh also obtained a high concentration in duck eggs (0.32 mg/kg w.w.). Shaheen et al.[32] from Bangladesh and Sobhan Ardakani et al.[53] from Iran recorded similar levels in chicken eggs. Hashish et al.[56] also found similar concentrations in hens' eggs from southern and middle Egypt (0.17 mg/kg w.w.). Leblanc et al.[54] from France and Ysart et al.[55] from United Kingdom reported the lowest concentrations (0.011; 0.003 mg/kg w.w., respectively). The results of the various studies analyzed show that the study region and poultry species greatly influence the levels of these trace elements in eggs.

Concentrations of As, Cd and Pb in Milk

For each trace element; the concentrations in milk differ according to the type of milk (cow, Ewe, goat) and for the same type of milk, the concentrations also differ according to the region of study. For As, Castro-González et al.[58] from Mexico obtained a significantly high concentration (1.15 mg/kg w.w.). Sarkar et al.[59] from India and Shaheen et al.[32] from Bangladesh also reported high concentrations in cow milk (1 and 0.44 mg/kg w.w., respectively). Monteverde et al.[60] registered the lowest and negligible values in two groups of cow milk in Italy whish are below the detection limit.

Reagrding Cd in the cow milk *(Table 4)*, the highest

concentration (1.24 mg/kg w.w.) was reported by Elatrash et al.[61] in Lybia. Monteverde et al.[60] in Italy; Ali et al.[62] in Tanzania; Ismail et al.[63] in Pakistan; Belete et al.[64] from Ethiopia and Bali from Algeria reported the lowest values of Cd. For Cd in goat milk, Balli et al.[65] registered different concentrations at three study sites in Algeria where the concentration recorded in site 1 was similar to that reported by Yabrir et al.[66] in goat milk (0.01 mg/kg w.w.). Yabrir et al.[66] also registered a low concentration in ewe milk (0.06 mg/kg w.w.).

For Pb; Malhat et al.[67] from the four sites in Egypt and Capcarova et al.[68] from Slovakia, and Elatrash et al.[61] in Lybia registered a high concentrations in cow milk while the highest values were reported by Malhat et al.[67] in Egypt *(Table 4)*. Ismail et al.[63] from Pakistan and Castro-González et al.[69] from Mexico registered the same value (0.03 mg/kg w.w.). The lowest concentrations of Pb in cow milk were registered by Belete et al.[64] from Ethiopia; Shaheen et al.[32] from Bangladesh; Ismail et al.[63] at site 3 from Pakistan; and Elsaim et al.[70] from Sudan. In Algeria, Yabrir et al.[66] registered different concentrations among which the value registered in the ewe milk was higher (1.18 mg/kg w.w.) than that measured in goat milk (0.07 mg/kg w.w.). For Pb in goat milk Balli et al.[65] recorded the highest values at three sites in Algeria. Homayonibezi et al.[71] found Pb values from the two Iranian sites that were different and low *(Table 4)*. The lowest values of Pb were registered by Ismail et al.[63] in four sites from Pakistan. All these results again show the importance of the animal species, study location and geographical area in the concentration of the toxic trace elements in milk.

Comparison of the Concentrations Obtained in Different Foodstuffs Reviewed with the Regulatory Limits

For As, there is no threshold limit set for this element in the foods analyzed. For Cd and Pb, the results of the studies were compared to the different regulatory limits set by the European Union (EU), Food Standards Australia New Zealand (FSANZ) and Joint FAO/WHO Expert Committee on Food Additives (JECFA), with

Table 4. *Summary of the concentrations of trace elements in milk*

Trace Elements	Type of Milk	Concentrations (mg/kg wet weight)	Contries		References
As	Cow milk	0.44	Bangladesh		Shaheen et al.[32]
		1.00	India		Sarkar et al.[59]
		0.12	Mexico		Castro-González et al.[69]
		1.15	Mexico		Castro-González et al.[58]
		BDL	Group 1	Italy	Monteverde et al.[60]
		BDL	Group 2		
Cd	Cow milk	0.03	Algeria		Bousbia et al.[72]
		0.27	Slovak		Capcarova et al. [68]
		0.44	Bangladesh		Shaheen et al.[32]
		0.08	Egypt		Enb et al.[73]
		0.28	Site 1	Egypt	Malhat et al.[67]
		0.27	Site 2		
		0.20	Site 3		
		0.22	Site 4		
		0.23	Site 5		
		0.00	Site 1	Sudan	Elsaim et al.[70]
		0.00	Site 2		
		0.00	Site 3		
		BDL	Group 1	Italy	Monteverde et al.[60]
		BDL	Group 2		
		ND	Tanzania		Ali et al.[62]
		1.24	Libya		Elatrash et al.[61]
		0.00	Site 1	Pakistan	Ismail et al.[63]
		BDL	Site 2		
		BDL	Site 3		
		0.00	Site 4		
		ND	Ethiopia		Belete et al.[64]
	Goat milk	0.01	Site 1	Algeria	Balli et al.[65]
		0.00	Site 2		
		0.03	Site 3		
		BDL	Site 1	Pakistan	Ismail et al.[63]
		BDL	Site 2		
		BDL	Site 3		
		0.001	Site 4		
	Ewe milk	0.06	Algeria		Yabrir et al.[66]
	Goat milk	0.01			

Table 4. *Summary of the concentrations of trace elements in milk (continued)*

Trace Elements	Type of Milk	Concentrations (mg/kg wet weight)	Contries		References
Pb	Cow milk	3.8	Slovak		Capcarova et al.[68]
		0.26	Tanznia		Ali et al.[62]
		0.03	Site 1	Pakistan	Ismail et al.[63]
		0.02	Site 2		
		BDL	Site 3		
		0.01	Site 4		
		0.27	Bangladesh		Shaheen et al.[32]
		3.43	Libya		Elatrash et al.[61]
		0.03	Mexico		Castro-González et al.[69]
		0.03	Mexico		Castro-González et al.[58]
		ND	Ethiopia		Belete et al.[64]
		0.06	Egypt		Enb et al.[73]
		1.85	Site 1	Egypt	Malhat et al.[67]
		3.5	Site 2		
		2.9	Site 3		
		4.4	Site 4		
		3.05	Site 5		
		0.00	Site 1	Sudan	Elsaim et al.[70]
		0.00	Site 2		
		0.00	Site 3		
	Ewe milk	1.18	Algeria		Yabrir et al.[66]
	Goat milk	0.07			
	Cow milk	0.02	Group 1	Italy	Monteverde et al.[60]
		0.03	Group 2		
	Goat milk	0.42	Site 1	Algeria	Balli et al.[65]
		0.38	Site 2		
		0.33	Site 3		
		0.00	Site 1	Pakistan	Ismail et al.[63]
		0.00	Site 2		
		0.00	Site 3		
		BDL	Site 4		
		0.14	Site 1	Iran	Homayonibezi et al.[71]
		BDL	Site 2		

As: Arsenic, Cd: Cadmium, Pb: Lead, BDL: Below Detection Limit, ND: Not detected

which the majority of the results largely exceeded these limits. As shown in *Table 1,* Uluozlu et al.[24] (6.09 mg/kg w.w.), Shaheen et al.[32] (0.23 mg/kg w.w.), Abduljaleel et al.[27] (0.15 mg/kg w.w.), Kamaly and Sharkawy [25] (5.87; 9.63; 13.01; 14; 10.93; 10.01 mg/kg w.w.), El Bayomi et al.[26] (0.06; 0.09 mg/kg w.w.) reported Cd concentrations in chicken meat exceeding the threshold limit set by EU [74] (0.05 mg/kg w.w.). In liver of poultry the concentration of Cd recorded by Uluozlu et al.[24] (2.24 mg/kg w.w.) largely exceed the threshold limit set by EU [74] (0.5 mg kg^1 w.w.) but the value they obtained in Kidney (0.25 mg/kg w.w.) is less the threshold limit (1 mg/kg w.w.) *(Table 4)*. For Cd in meat of cattle; the values reported by Kasozi et al.[40] (0.4 mg/kg w.w.), Nkansah and Ansah [43] (0.079 mg/kg w.w.), Sathyamoorthy et al.[10] (6.6; 5.1 mg/kg w.w.), Adjei et al.[35] (0.1 mg/kg w.w.), Shaheen et al.[32] (0.57 mg/kg w.w.) all exceeded the limits set by EU [74] and FSANZ [75] (0.05 mg/kg w.w.). In the meat of sheep; Darwish et al.[42] (0.06 mg/kg w.w.), Raeeszadeh et al.[39] (0.23 mg kg^1 w.w.), Abd-Elghany et al.[36] (0.30 mg/kg w.w.) recorded the highest concentrations which exceeded the limits set by EU [74] and FSANZ [75] (0.05 mg/kg w.w.). There are no threshold limits for Cd in eggs and milk. For the concentrations of Pb in meat of poultry *(Table 1);* in all studies concentations were higher than the permissible limits (0.1 mg/kg w.w.) set by EU [74]; FSANZ [75] and by JECFA [76] except those registered by Yilmaz and Gecgel [33] and Kamaly and Sharkawy [25]. In the liver of poultry the values recorded by Kamaly and Sharkawy [25] in differents brands of poultry (2.57; 3.15; 2.56; 2.58; 3.30; 5.55) largely exceeded the permissibl limit set by EU [74] (0.1 mg/kg w.w.). In the meat of cattle the majority of the concentrations of Pb greatly exceeded the permissible limit *(Table 5),* e.g. Kasozi et al.[40] (5.4 mg/kg w.w.), Chowdhury et al.[41] (4.62 mg kg^1 w.w.), Nkansah and Ansah [43] (1.15 mg/kg w.w.), Sathyamoorthy et al.[10] (4.6; 2.4 mg/kg w.w.), Shaheen et al.[32] (0.48 mg/kg w.w.) *(Table 2)*. In the meat of sheep the concentrations recorded by Darwish et al.[42] (0.25 mg/kg w.w.),

Table 5. Permissible limits in meat, poultry, eggs and milk

Trace Elements	Type of Food	Permissible Limits (mg/kg wet weight)	References
Cd	Meat of bovine animals, sheep and poultry	0.05	EU [74]
	Liver of poultry	0.5	EU [74]
	Kidney of poultry	1	EU [74]
	Meat of cattle and sheep	0.05	FSANZ [75]
Pb	Meat of bovine animals, sheep and poultry	0.10	EU [74]
	Meat of cattle, sheep and poultry	0.10	FSANZ [75]
	Meat of cattle, sheep and poultry	0.10	JECFA [76]
	Poultry offal	0.1	EU [74]
	Milk	0.02	EU [74]
		0.02	JECFA [76]

As: Arsenic, Cd: Cadmium, Pb: Lead, EU: Commission Regulation, FSANZ:Food Standards Australia New Zealand.JECFA: Joint FAO/WHO Expert Committee on Food Additives

Raeeszadeh et al.[39] (11.79 mg/kg w.w.), Abd-Elghany et al.[36] (0.48 mg/kg w.w.), Shaheen et al.[32] (1.15 mg/kg w.w.) far exceeded the permissible limits set by EU [74]; FSANZ [75] and by JECFA [76] (0.1 mg/kg w.w.). In the cow, goat and ewe milk the majority of the results *(Table 4)* exceed the permissible limit set by EU [74] and JECFA [76] (0.02 mg/kg w.w.). Capcarova et al.[68] (3.8 mg/kg w.w.), Ali et al.[62] (0.26 mg/kg w.w.), Shaheen et al.[32] (0.27 mg/kg w.w.), Ismail et al.[63] (0.03 mg/kg w.w.), Elatrash et al.[61] (3.43 mg kg^{1} w.w.), Castro-González et al.[58] and Castro-González et al.[69] (0.03 mg/kg w.w.), Enb et al.[73] (0.06 mg kg^{1} w.w.), Malhat et al.[67] (1.85; 3.5; 2.9; 4.4; 3.05 mg/kg w.w.), Monteverde et al.[60] (0.03 mg/kg w.w.) in cow milk. Yabrir et al.[66] (1.18; 0.07 mg/kg w.w.) in ewe and goat milk, respectively. Balli et al.[65] (0.42; 0.38; 0.33 mg/kg w.w.), Homayonibezi et al.[71] (0.14 mg/kg w.w.) recorded a values which largely exceed the permissible limit *(Table 5)*. No permissible limits are set for Pb in eggs.

Conclusion and Recommendation

In this review we have thoroughly analyzed and discussed the data of different studies on the contamination of certain foodstuffs of animal origin by three toxic trace elements (As, Cd and Pb), and the results have clearly identified the the presence of contamination in these foodstuffs by these trace elements. In the case of arsenic, the ofe highest concentrations have been recorded in cattle meat (5.6, 3.7 mg/kg w.w.). For Cd the highest concentrations were recorded in the meat of chicken and the meat of cattle (6.09 and 6.6, mg/kg w.w., respectively). The highest concentration of Pb was recorded in the meat of sheep (11.7 mg/kg w.w.). Surprisingly the majority of the concentrations of the three trace elements studied found in all these food matrices largely exceed the international regulatory thresholds which points to a potential hazard risk for the health of the consumer. To alleviate this potential international public health problem related to trace element poisoning via food, it will be very important for researchers in this field to make further assessments of this subject and to identify, if possible, the source of contamination for these foodstuffs by these contaminants by carrying out studies on water and food intended for the animals, and also to carry out regular monitoring and controls in order to prevent the risks to populations consuming these local foods.

Declarations

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Research Article

Application of A Rational Feeding Strategy to Increase the Cell Density of Avian *Pasteurella multocida*

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Abstract

Avian *Pasteurella multocida* infection, can cause serious economic losses to the poultry industry every year. The inactivated vaccines of avian *Pasteurella multocida* are used to prevent infection. Increasing the cell density of avian *Pasteurella multocida* is the key to the application of these inactivated vaccines. This can be achieved by controlling the feeding strategy. This study aimed to achieve high-cell-density cultivation of avian *Pasteurella multocida* by applying an appropriate medium and a rational feeding strategy based on viable cell growth and dissolved oxygen level variation during a fermentation process. An optimized medium suited for the growth of avian *P. multocida* was used. Meanwhile, besides the online real-time determination of viable cell density, the concentration of glucose was maintained at 1.5 g/L using a glucose-stat feeding strategy after 2 h. The selected nutrient mixture, including yeast extract, tryptone, betaine, V_{B1}, and V_H, was fed using a dissolved oxygen feedback feeding strategy after 4 h. As a result, the viable cell density and cell count of avian *P. multocida* under optimized conditions were increased to OD_{600}: 9.38 and 4.58×10^{10} CFU/mL, which were higher by 7.27 and 7.26 times than those under the original conditions, respectively.

Keywords: Avian *Pasteurella multocida,* Cell density, Feeding strategy, Medium, Online viable cell monitoring

Introduction

Pasteurella multocida, a Gram-negative bacterium, is a zoonotic pathogen [1,2]. Fowl cholera, caused by avian *P. multocida*, is a highly contagious disease among various domestic and wild avian species. It is associated with high mortality and morbidity, resulting in significant economic losses in global livestock production [3]. Antibiotics used to treat diseases caused by avian *P. multocida* often result in the existence of antibiotic residues in animal-derived products, eventually leading to antibiotic resistance [4,5]. Vaccination is a successful method of controlling avian *P. multocida* infection, but vaccine application and disease control are limited by low fermentation concentration and high production costs [6,7]. The inactivated vaccines of avian *P. multocida* have been proven effective in preventing avian *P. multocida* infection [8]. Increasing the cell density of avian *P. multocida* can reduce vaccine production costs and improve vaccine efficacy.

Appropriate concentrations of nutrients in the fermentation medium could satisfy the requirement of cell growth and increase cell density [9]. Optimizing the medium components via a Box-Behnken design, the cell count of *Lactobacillus plantarum* Y44 was increased to 3.363×10^{10} CFU/mL, which was higher by 6.11 times than that in the MRS medium [10]. Meanwhile, based on the characteristics of bacterial growth, the addition of specific nutrient components could further increase the abundance of bacteria. Further, 20 mM histidine was added to the basal medium, which greatly enhanced the growth of *gdhA* derivative *P. multocida* B:2 mutant by approximately 19 times compared with that in the control culture [11]. Thiamine and biotin had the highest bioavailability from midexponential to late-exponential phase of *Streptococcus thermophilus* MN-ZLW-002, media containing trace elements can be useful for high-density cultures of bacteria [12]. Betaine can act as a stress

protectant, methyl donor, or enzyme stabilizer *in vitro* [13], the maximum L-threonine production of 127.3 g/L and glucose conversion percentage of 58.12% was obtained fed by *Escherichia coli* with the glucose solution containing 2 g/L betaine hydrochloride, which increased by 14.5 and 6.87% more compared to that of the control [14].

In the present study, appropriate nutrient components were selected for the growth of avian *P. multocida*. Meanwhile, the time point of feeding was detected rationally based on online viable cell monitoring. An appropriate feeding strategy was used to maintain glucose concentration and feed nutrient mixture, leading to an increase in the cell density of avian *P. multocida.*

Material and Methods

Strain and Media

The strain of avian *P. multocida* used in this study was obtained from the China Veterinary Culture Collection Center (Collection Number: CVCC44802).

The strain was cultured in the TSB medium containing (in g/L) glucose 2.5, tryptone 17.0, soy peptone 3.0, K_2HPO_4 2.5, and NaCl 5.0.

The four kinds of media for avian *P. multocida* fermentation in *Table 1*. The five kinds of nutrient mixture solutions used as the feed medium during avian *P. multocida* fermentation in *Table 2*.

Culture Conditions

A sample of 100-mL seed medium in a 500-mL baffled flask was inoculated with a single colony of avian *P. multocida* and cultivated at 37°C by shaking at 160 rpm for 8 h. This culture grown in the baffled flask was then inoculated aseptically (5% *v*/*v*) into 3 L of fermentation medium in a 5-L fermenter (Biotech 2012, Shanghai Bailun Biotech Co., Ltd., Shanghai, China). The temperature and pH were controlled at 37°C and 7.0, respectively, whereas the DO level was maintained at 30% by adjusting the agitation and aeration rates. We maintained the concentration of glucose at the stated level and fed the nutrient mixture solution using the DO feedback feeding strategy [15] to meet the experimental requirements.

Analytical Methods

The viable cell density of avian *P. multocida* was assessed by combining cell capacitance determination with spectrophotometric absorbance reading (OD_{600}). The cell capacitance was determined using an online viable cell monitoring system (DN 12-120, Switzerland), in a high-frequency electric field, living cells are treated as tiny capacitors, according to the principle of capacitance measurement a capacitance probe is used to measure the charge carried by the viable cell count. The viable cell count was determined by a flat colony counting method, dilution of the culture solution to the Appropriate concentration and apply to glass garden with 0.1 mL, repeat three times; calculated from the equation between capacitance and the viable cell count. The glucose concentration was determined using a glucose biosensor (SBA-40E; Biology Institute of Shandong Academy of Sciences, China), glucose oxidation to generate H_2O_2, H_2O_2 contact with the electrode generates a current, the current signal is proportional to the concentration of glucose, used to indirectly determine the concentration of glucose ($Glucose + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$).

Table 1. *The media for avian P. multocida fermentation*

Media	Components of Media (g/L)
Medium I	glucose 5.0, yeast extract 5.0, tryptone 10.0
Medium II	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0
Medium III	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0
Medium IV	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0, betaine 2.0, V_{B1} 0.025, V_H 0.01.

Table 2. *The nutrient mixture solutions for avian P. multocida fermentation*

Supply Strategy	Components of Nutrient Mixture Solution (g/L)
Strategy I	yeast extract 5.0 and tryptone 10.0
Strategy II	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0
Strategy III	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, and KCl 2.0
Strategy IV	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0, betaine 2.0, V_{B1} 0.025, and V_H 0.01
Strategy V	yeast extract 5.0, tryptone 10.0, betaine 2.0, V_{B1} 0.025, and V_H 0.01

Result

The Relationship of Between Capacitance Value, Viable Cell Density and Cell Count for Avian *P. multocida*

The relations among capacitance value, cell density, and viable cell counts during the logarithmic phase of avian *P. multocida* cultivation are displayed in *Fig. 1*. The cell count and capacitance showed a good linear relationship (y=8.0597x; y: cell count OD_{600}; x: capacitance pF/cm; R^2=0.9922); the cell count and viable cell density showed a good linear relationship (y=1.9241x; y: cell count OD_{600}; x: viable cell density (x10^{10}CFU/mL); R^2=0.9934); the capacitance and viable cell density showed a good linear relationship (y=4.3469x; y: capacitance pF/cm; x: viable cell density (x10^{10}CFU/mL); R^2=0.9906). The results showed a good linear relationship between capacitance value, viable cell density and cell count. The viable cell density during avian *P. multocida* cultivation, thus, could be calculated based on viable cell density via this equation.

Avian *P. multocida* Fermentation with Different Media

The cell capacitance and glucose concentration with different media in avian *P. multocida* fermentation are displayed in *Fig. 1*. The type of medium not only affected cell growth according to cell capacitance but also impacted glucose consumption. The cell capacitance with medium IV was the highest compared with that with other media. The maximum cell capacitance with medium IV was 2.0 h, indicating that the cells of avian *P. multocida* did not grow after 2.0 h. The glucose consumption with medium IV was the maximum, which was caused by higher cell density. The glucose concentration was 1.0 g/L after cultivation for 2.0 h.

Viable Cell Density and Cell Count

The viable cell density and cell count with different media during avian *P. multocida* fermentation are presented in *Fig. 2*. The viable cell density and cell count increased with an increase in the amount of nutrients in the media, which was in accordance with the cell capacitance shown in *Fig. 1*. The viable cell density (2.58, OD_{600}) and cell count ($1.26^{x}10^{10}$ CFU/mL) with medium IV were the highest, which were higher by 2.01 and 2.03 times than those with medium I.

Avian *P. multocida* Fermentation with Different Glucose Concentrations

Fig. 3 shows the cell capacitance, viable cell density, and cell count with different glucose concentrations after 2.0

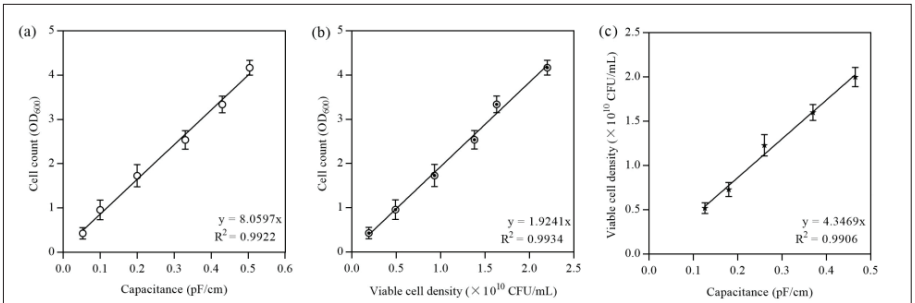

Fig 1. Relationship between capacitance and cell density and between viable cell density and cell count. (a) Linear relationship between capacitance and cell count. (b) Linear relationship of between viable cell density and cell count, (c) Linear relationship of between capacitance and viable cell density

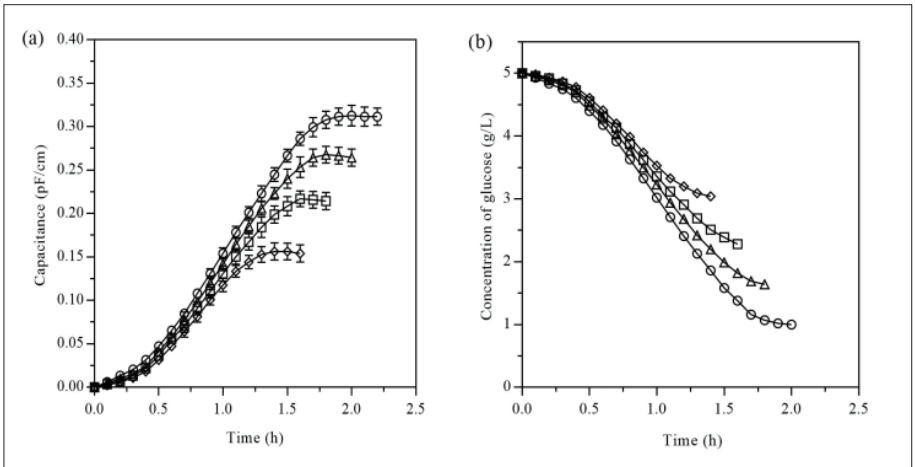

Fig 2. Cell capacitance (a) and glucose concentration (b). All values are presented as the mean±SD

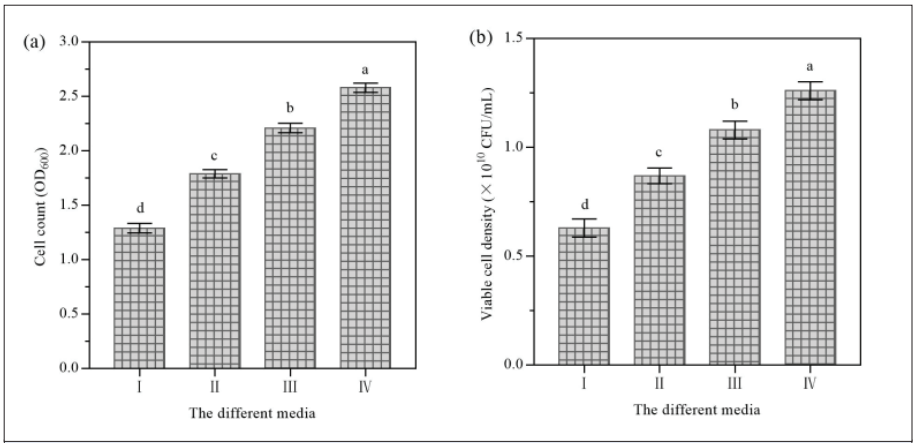

Fig 3. Effect of different media on cell count (a) and viable cell density (b) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicate significant difference (P<0.05)

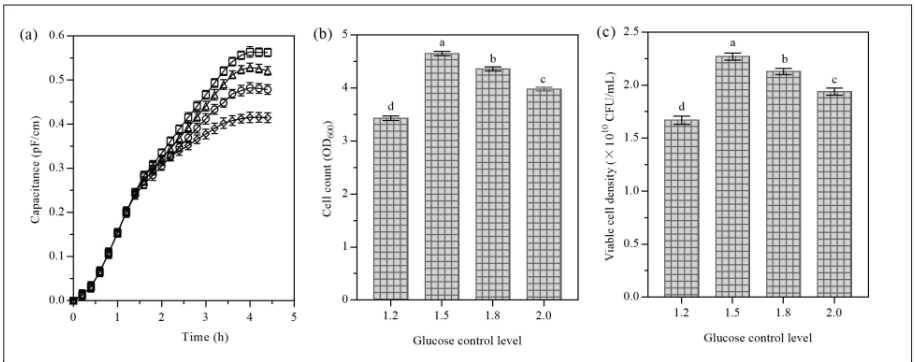

Fig 4. Effect of glucose control strategy on cell capacitance (a), cell count (b) and viable cell density (c) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicatesignificant difference (P<0.05)

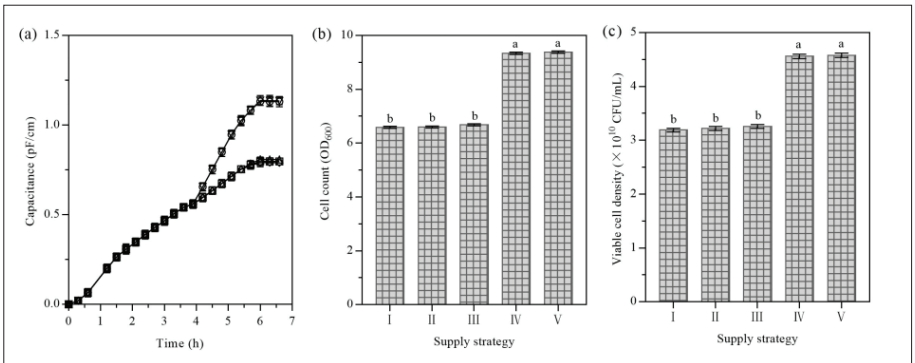

Fig 5. Effect of feed nutrient mixture solutions on cell capacitance (a), cell count (b) and viable cell density (c) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicate significant difference (P<0.05)

h using the glucose-stat feeding strategy. The glucose concentration controlled at more than 1.0 g/L could extend the growth period of avian *P. multocida* and increase its viable cell density and cell count, but the viable cell density and cell count decreased with glucose concentration more than 1.5 g/L. The maximum cell capacitance was achieved at a glucose concentration of 1.5 g/L, and the DO level increased suddenly at the same time. The viable cell density and cell count obtained at the aforementioned glucose concentration were the highest, which were 4.65 (OD_{600}) and 2.27×10^{10} CFU/mL, respectively *(Fig. 4)*.

Avian *P. multocida* Fermentation with Different Feed Mixture Solutions

The avian *P. multocida* fermentation with different nutrient mixture solutions after 4.0 h using the DO feedback feeding strategy is displayed in *Fig. 5*. The nutrient mixture solutions supplied after 4.0 h also extended the

cell growth period, leading to an improvement in the viable cell density and cell count. The viable cell density and cell count with the feed nutrient mixture I were 6.53 (OD_{600}) and 3.19×10^{10} CFU/mL. The viable cell density and cell count with feed nutrient mixture II and mixture III were not significant different from those with feed nutrient mixture I. The viable cell density and cell count with feed nutrient mixture IV increased by 43.03% and 42.95% compared with that with feed nutrient mixture I. The viable cell density and cell count with feed nutrient mixture V were 9.38 (OD_{600}) and 4.58×10^{10} CFU/mL, respectively, which were not significantly different from those with feed nutrient mixture IV.

Discussion

This study showed that the growth of bacteria was affected by fermentation medium and cultivation conditions. The form of the medium was the basis for fermentation [16]. Higher cell density and cell count were obtained with the enriched medium compared with different media for avian *P. multocida* fermentation; the highest cell density and count were achieved with medium IV. During *Streptococcus suis* fermentation, the cell density increased by 15.37% on increasing the concentration of nitrogen source [17].

The key nutrient compounds were essential for cell growth and enhancing cell density in the medium [18]. As shown in *Fig. 5*, yeast extract, tryptone, betaine, V_{B1}, and V_H increased the cell density and viable cell count. The hydrolyzed yeast components contained in yeast extract efficiently supplied the TCA cycle, improving oxidative metabolism and increasing Chinese hamster ovary cell density by 70% [19]. Adding an appropriate amount of V_H improved the cell viability of *Corynebacterium glutamicum*, thus increasing the yield and productivity of the product [20]. When betaine was fed into the fermentation culture medium, the metabolic flux entering into the pentose phosphate pathway and the biosynthesis route of L-threonine increased by 57.3% and 10.1%, respectively, increasing the production of L-threonine by 14.5% [21].

The concentration of glucose affected cell growth, and a higher cell density of avian *P. multocida* was achieved at a glucose concentration of 1.5 g/L after 2.0 h. When the concentration of glucose was less than 1.5 g/L, the glucose uptake rate of cells was not adequate for the growth of avian *P. multocida* [22,23]. However, the synthesis of by-products inhibited the growth of *E. coli* [24]. High glucose concentrations can increase the osmotic pressure of the culture medium, which is detrimental to microbial growth, thus, the growth of avian *P. multocida* was inhibited at a glucose concentration of more than 1.5 g/L. Glucose concentration was a key parameter for the biosynthesis of desired products by *E. coli* [25]. The flux and accumulation of acetate during L-tryptophan production were reduced by genetically modifying a strain that produced L-tryptophan and implementing glucose feedback feeding. The flux of tryptophan formation and yield of L-tryptophan increased by 21.36% and 35.81%, respectively [26].

The viable cell sensor was a reliable online biomass monitoring tool for Gram-positive and Gram-negative bacteria [27]. Applying a rational feeding method was an effective strategy for high-cell-density cultivation of bacteria [28]. Based on the selection of feeding time by online viable cell monitoring, suitable nutrients were fed using the appropriate feeding strategy after 2 or 4 h, leading to high-cell-density cultivation of avian *P. multocida*. The viable cell sensor and electronic nose in ethanol fermentation is used to monitor the cell density of *Saccharomyces cerevisiae* B1, a dynamic feeding strategy of glucose was applied, and the ethanol concentration, productivity, and yield were enhanced by 15.4%, 15.9%, and 9.0%, respectively [28]. Real-time monitoring and pH regulation strategies were implemented. The total viable count of *Clostridium butyricum* was 3.32 times higher than that in the control group, and the sporulation rate escalated from 73.9% to 96.6% [29]. In *Glaesserella parasuis* (*G. parasuis* formerly *Haemophilus parasuis*) fermentation, based on online viable cell monitoring, combined with the relationship between nicotinamide adenine dinucleotide (NAD) consumption and cell growth, the feeding concentration of NAD was adjusted to 30 mg/L. The viable cell count of *G. parasuis* increased to 1.57×10^{10} CFU/mL, which was higher by 8.26 times compared with that obtained in tryptic soy broth (TSB) medium [30]. Real-time monitoring allows for a more comprehensive understanding of the bacterial fermentation process. The development and application of effective real-time and online sensors plays an important role in optimizing biological processes to improve product concentration, productivity, and yield [30], thus providing a more effective feeding strategy for bacterial fermentation culture.

In this study, an appropriate medium was selected for the fermentation of avian *P. multocida*. Meanwhile, using online real-time monitoring and DO-level variations, the glucose concentration was maintained at 1.5 g/L after 2 h and a nutrient mixture containing yeast extract, tryptone, betaine, V_{B1}, and V_H was fed after 4 h using the DO feedback feeding strategy. Thus, the viable cell density and cell count of avian *P. multocida* under the optimized conditions increased to OD_{600}: 9.38 and 4.58×10^{10} CFU/mL, which were higher by 7.27 and 7.26 times than those under the original conditions, respectively. This experiment provides a reference for the high-density fermentation culture of avian *P. multocida* and lays the foundation for the preparation of inactivated vaccine. Simultaneously, the research methods presented in this study provide a

theoretical foundation and technical approach for the high-density cultivation of other auxotroph bacteria, only the media components and media replenishment strategies were investigated and the effect of fermentation process parameters on avian *P. multocida* was neglected.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (L.C.).

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Authors' Contributions: YS and YB performed the experiments, analysed the results, and drafted the manuscript. XZ, LG and WW assisted in the experimental design and summarized the experimental results. QF, NT and CW put forward valuable suggestions for the revision and improvement of the paper. XX, JL and LC conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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Research Article

Impact of Vitamin E and Selenium Prior the Ovsynch Synchronization on Reproductive Performance in Friesian Dairy Cows During Hot Season

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Abstract

Heat stress during hot season enhances oxidative stress, alters hormonal secretion and adversely affects reproductive performance. So, it's necessary to supply antioxidants such as vitamin E and selenium. This work aimed to investigate the impact of vitamin E and selenium administration on reproductive performance before ovsynch synchronization and artificial insemination (AI) in Friesian cows during hot season. Twenty ovsynch-synchronized Friesian cows were divided according to the time of vitamin E and selenium administration into four groups (each 5 cows), G_1 control group without any administration, G_2 administered pre-synchronization, G_3 administered at AI, G_4 administered two doses pre-synchronization and at AI. Follicular functions during synchronization period revealed a significant increase in both follicle number and diameter of G_2 and G_4 compared to G_1 and G_3. Blood samples were collected from all cows weekly from 0-day to 5th week post AI for hormones and antioxidant detection. Serum analysis results revealed increased progesterone, prolactin, CAT, SOD, GSH and total antioxidant capacity (TAC), while decreased cortisol and MDA of G_4 compared to G_1, G_2 and G_3. Pregnancy detection was performed at 60 days post-AI. Conception rates were 40, 60, 60, and 80% in G_1, G_2, G_3 and G_4, respectively. Therefore, vitamin E and selenium administration improve antioxidant activities and overcome oxidative stress providing a better impact on reproductive performance even in hot season.

Keywords: Antioxidants, Conception rate, Heat stress, Hormones, Synchronization, Vitamin E and selenium

Introduction

Heat stress during hot season could change the physiological, biochemical, and productive functions of the livestock resulting in reduced fertility and economic losses for the dairy industry. Furthermore, the adverse effect of environmental high temperatures on fertility rates cannot be omitted [1]. Exposure of dairy cows to heat stress during early lactation can adversely affect their productivity, fertility, and blood biochemistry in subsequent lactation periods [2]. Under such conditions, heat stress can affect the cellular functions of germ cells and directly impact fertility. Heat stress directly affects follicular development, follicular waves, steroid gene activity in both follicular and granulosa cells, corpus luteum development and functionality via reduced progesterone production [3]. Low progesterone secretion during the luteal phase can alter oocyte maturation leading to implantation failure and early embryonic death [4]. While, reduced feed intake during heat stress reduces the frequency of luteinizing hormone (LH) and lengthens the follicular waves with the appearance of smaller dominant follicles [5].

Heat stress increases body temperature, pulse rate, and respiration rate leading to reduced feed intake, redistribution of blood flow, weakening of the immune system, and changes in endocrine function, which ultimately affects fertility [6]. Environmental heat stress, may

Table 1. *Average of the daily ambient temperature (°C), relative humidity (%), and temperature humidity index (THI) during the hot season*

Month	Ambient Temperature (°C)			Relative Humidity (%)			THI
	Max.	Min.	Average	Max.	Min.	Average	
June	33	26	28.89±0.28	57	35	50.85±0.90	76.82±0.27
July	32	27	29.85±0.21	59	42	51.14±0.62	78.16±0.25
August	33	29	29.95±0.16	60	45	54.04±0.76	78.74±0.16
September	31	26	28.51±0.18	60	47	54.57±0.72	76.92±0.28

Data are expressed as average ± SE

activate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal axis, both of which mediate hormonal changes. Additionally, it suppresses thyroid and pancreas activity, resulting in decreased secretion of thyroid hormones and insulin, while concurrently enhancing adrenal cortex activity and increasing the secretion of the stress hormone cortisol [7]. Animals exposed to heat stress revealed altered hormones concentrations such as thyroxin, cortisol, and prolactin. Plasma cortisol levels are used as a marker of heat stress, decreasing during heat acclimation to reduce heat production [8]. Furthermore, heat stress reduces antioxidant activity and induces oxidative stress, while it promotes excessive production of free radicals, and reduces antioxidant status by impairing antioxidant defense systems [9].

Vitamin E and selenium (Se), both necessary co-factors of the enzyme glutathione peroxidase (GSH-px), are a vital part of the antioxidant defense system present in various cell types [10], and are playing important roles in animal growth performance, immune function, and reproductive performance through their participation in key enzymatic reactions [11]. The contribution of vitamin E and Se to progesterone production by the corpus luteum improves the resumption of ovarian activity [12]. It has been demonstrated that administration of vitamin E and/or Se increases the pregnancy rates and both together can improve the conception rate of cattle and sheep by decreasing early embryonic deaths [13,14]. Furthermore, vitamin E and Se could improve reproduction, metabolic profiles, and antioxidant capacity in cows under conditions of heat stress.

The purpose of this study was to investigate the impact of vitamin E and selenium administration on the reproductive performance of Friesian cows during the hot summer season.

Material and Methods

Ethical Statement

This protocol was approved by the Research Committee of the Animal Health Research Institute and authorized by the Institutional Animal Care and Use Committee (ARC-IACUC)/Agricultural Research Center (ARC/AHRI/33/24).

Farm Location and Climatic Conditions

This work was established at Animal Production Experimental Station, Sakha (31°05'17.3" north and 30°56'29.9" east), Kafr El-Sheikh, belonging to Animal Production Research Institute (APRI), Agricultural Research Center, Egypt from June to September 2022. Ambient temperature and relative humidity (RH) during the hot season are shown in *Table 1*. These parameters were recorded daily for the entire experimental period outside by the National Aeronautics and Space Administration (NASA) Langley Research Centre (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program. Temperature humidity index (THI) was calculated using the formula proposed by Mader et al.[15]:

THI = (0.8 × ambient temperature) + [(% RH) /100) × (ambient temperature – 14.4)] + 46.4

Armstrong [16] describes the temperature humidity index (THI) for dairy cattle, establishing a spectrum where a THI up to 71 is classified as comfort. Indices ranging from 72 to 79 signify mild thermal stress, 80 to 89 indicate moderate stress, and values exceeding 90 represent severe stress, and a THI surpassing 100 indicates lethal outcomes.

Experimental Design

Twenty apparently healthy Friesian cows, with an age range 5-6 years, an average body weight 500±45 kg, daily milk yield 18±5 kg, and within 55 to 70 days postpartum, were used in this study. Ovsynch synchronization of all cows was performed according to the following protocol; 2 mL GnRH was injected on day 0, followed by 2 mL $PGF_2\alpha$ injected on the 7th day, and concluding with a second 2 mL GnRH injection on day 9 with artificial insemination (AI) being performed 16 hours post-final injection [17]. The cows were divided into four groups; the 1st group (N=5) did not receive any administration used as a control group (G_1), the 2nd group (N=5) received a single dose of E-SELEN® (1 mL/45 kg BW) immediately pre-synchronization only (G_2), the 3rd group (N=5) was administered a single dose of E-SELEN® (1 mL/45 kg BW) at the time of AI only (G_3), and the 4th group (N=5) received a double dose of

E-SELEN® (1 mL/45 kg BW) the first dose immediately pre-synchronization and the second dose at the time of AI (G_4).

The follicular functions were detected on day 0, as well as on the 7th and 10th day after synchronization using a real-time ultrasound scanning device (Ultrasound scanner, model MyLab 30 Gold ultrasound machine-Esaote-Pie Medical-Holland-Italy) with LV 315 Linear trans-rectal probe. Scans of both ovaries were executed to enumerate follicles and measure their diameters in millimeters. Also, the same real-time ultrasound scanning device was used for pregnancy diagnosis on day 60 post-AI.

Samples

Blood samples were collected from the jugular vein of all cows on day 0, and 1st, 2nd, 3rd, 4th, 5th-week post-AI. Serum samples were obtained by centrifugation of blood samples at 5000 rpm for 5 min. The clear sera were aliquoted into clean, dry Eppendorf tubes and maintained at 4°C for subsequent hormonal assays, followed by storage at -20°C pending biochemical analyses.

Chemicals and Medications

Estrumate®; Beef and dairy cattle were intramuscularly injected with Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (MSD Animal Health Company). Cystorelin®; a synthetic GnRH analogue for the release of both luteinizing (LH) and follicle-stimulating hormone (FSH), contains Gonadorelin (GnRH) diacetate tetrahydrate 50 mcg/mL, manufactured by Ceva-Africa Company. E-SELEN®; each mL contains 5.48 mg sodium selenite (equivalent to 2.5 mg selenium), 50 mg (68 IU) vitamin E (as α-alpha tocopherol acetate), manufactured by NITA-FARM Company.

Serum Biochemical Analysis

Serum progesterone concentrations were estimated using ELISA kit (Catalog No. 10005, PerkinElmer) with a sensitivity threshold of 0.2 ng/mL. The serum concentration of tri-iodothyronine (T_3), thyroxin (T_4), and thyroid stimulating hormone (TSH) were assessed using ELISA kit (Immunospec corporation, USA, catalog No. PerkinElmer-10301, PerkinElmer-10302 and PerkinElmer-10304). Serum cortisol levels were evaluated using ELISA kit (PekinElmer-10005; 00000; DBC, Canada; catalog No. CAN-C-270 and SinoGene-Clon-SG-60105). Serum prolactin (PRL) concentration were detected using Bovine Prolactin ELISA kit (SinoGene-Clon Biotech Co., Ltd) with a sensitivity threshold of 0.3 ng/mL.

Commercial kits were used to determine Superoxide dismutase activity (BIODIAGNOSTIC, CAT. No. SD2521) and total antioxidant capacity (TAC) (CAT. No. TA2513). Lipid peroxidation expressed as malondialdehyde (MDA) was detected calorimetrically according to Okhawa et al.[18], reduced glutathione (GSH) levels were detected by the method described by Pleban et al.[19], and catalase (CAT) activity was detected by the method described by Aebi [20].

Statistical Analysis

Data were statistically analyzed using one-way (ANOVA) by SPSS 22 software to assess the significant differences between groups and times within groups. The results were demonstrated as means ± SE. The results were considered statistically significant at $P<0.05$.

Results

The results of follicular functions *(Table 2)* revealed a significant ($P<0.05$) increase in follicle number (FN) in both G_2 and G_4 groups on the 10th day of synchronization compared to the other groups. There was a gradual significant ($P<0.05$) increase in follicle diameter (FD) from the 7th day to the 10th day of synchronization in both G_2 and G_4 groups compared to groups G_1 and G_3.

Serum hormone results *(Table 3)* revealed a gradual increase of progesterone (P4) level in all groups, but there was a significant ($P<0.05$) increase in G_4 from the 1st week to week 5 post-AI compared to G_1 and a non-significant

Table 2. *Effects of vitamin E and selenium administration of ovsynch synchronized Friesian cows on follicle number (FN) and diameter (FD) during hot season*

Item	Time	Groups			
		1st Group (G_1)	2nd Group (G_2)	3rd Group (G_3)	4th Group (G_4)
Follicle Number	0-day	1.80±0.84	2.00±0.71	1.80±0.84	2.00±0.71
	7th day	2.00±0.71	3.00±0.71	2.20±0.84	3.20±0.84
	10th day	2.20 ± 0.84^{b}	3.40 ± 1.14^{a}	2.40 ± 0.55^{b}	3.60 ± 1.14^{a}
Follicle Diameter (mm)	0-day	5.60±0.87	6.26±0.71	5.66±0.64	6.14±0.86
	7th day	6.16 ± 0.66^{bB}	9.40 ± 0.81^{aAB}	6.22 ± 0.74^{bB}	9.62 ± 0.86^{aAB}
	10th day	7.82 ± 0.77^{bA}	10.00 ± 0.8^{aA} 7	$7.90\pm0.8\,9^{bA}$	10.60 ± 0.80^{aA}

Data are expressed as mean ± SE

[a,b] *Superscript: Mean significance difference among groups in the same row on P<0.05*

[A,B] *Superscript: Mean significance difference among administration times in the same column on P<0.05*

Table 3. *Effects of vitamin E and selenium administration of ovsynch synchronized Friesian cows on serum hormones during hot season*

Parameters	Groups	Times					
		0-day	Week 1	Week 2	Week 3	Week 4	Week 5
Progesterone (ng/mL)	1st group (G_1)	0.287 ± 0.05^{C}	1.037 ± 0.411^{bB}	2.580 ± 0.935^{A}	3.301 ± 1.724^{bA}	3.535 ± 1.861^{bA}	4.594 ± 2.201^{bA}
	2nd group (G_2)	0.311 ± 0.056^{D}	2.376 ± 0.704^{abC}	3.740 ± 1.340^{BC}	5.380 ± 1.81^{aAB}	5.580 ± 1.630^{abAB}	6.499 ± 1.446^{abA}
	3rd group (G_3)	0.294 ± 0.057^{D}	2.288 ± 0.571^{abC}	3.820 ± 1.150^{BC}	5.420 ± 1.85^{aAB}	5.620 ± 1.650^{aAB}	6.579 ± 1.495^{abA}
	4th group (G_4)	0.344 ± 0.072^{E}	2.592 ± 0.673^{aD}	4.420 ± 1.336^{CD}	6.420 ± 2.13^{aBC}	7.420 ± 2.023^{aAB}	8.379 ± 1.847^{aA}
T_3 (ng/mL)	1st group (G_1)	3.034 ± 0.417	2.993 ± 0.21^{b}	2.933 ± 0.201^{b}	2.994 ± 0.171^{b}	3.009 ± 0.193^{b}	3.088 ± 0.271^{c}
	2nd group (G_2)	3.165 ± 0.334^{B}	3.25 ± 0.233^{abAB}	3.411 ± 0.227^{aAB}	3.63 ± 0.294^{aA}	3.598 ± 0.245^{aA}	3.368 ± 0.275^{bcAB}
	3rd group (G_3)	3.032 ± 0.385^{C}	3.27 ± 0.329^{abBC}	3.59 ± 0.255^{aAB}	3.687 ± 0.264^{aA}	3.694 ± 0.205^{aA}	3.613 ± 0.232^{abAB}
	4th group (G_4)	3.294 ± 0.347^{B}	3.452 ± 0.328^{aAB}	3.71 ± 0.268^{aA}	3.763 ± 0.26^{aA}	3.783 ± 0.272^{aA}	3.86 ± 0.225^{aA}
T_4 (µg/dL)	1st group (G_1)	6.549 ± 0.689	6.233 ± 0.749	6.224 ± 0.632	6.396 ± 0.602	6.414 ± 0.57	6.444 ± 0.63
	2nd group (G_2)	6.494 ± 0.668	6.362 ± 0.597	6.28 ± 0.579	6.421 ± 0.644	6.542 ± 0.555	6.636 ± 0.532
	3rd group (G_3)	6.589 ± 0.727	6.433 ± 0.572	6.456 ± 0.694	6.441 ± 0.669	6.58 ± 0.568	6.676 ± 0.583
	4th group (G_4)	6.629 ± 0.668	6.808 ± 0.637	6.861 ± 0.496	6.804 ± 0.556	7.053 ± 0.479	7.118 ± 0.574
TSH (µU/mL)	1st group (G_1)	0.871 ± 0.068^{bD}	1.025 ± 0.089^{bC}	1.032 ± 0.058^{C}	1.057 ± 0.061^{BC}	1.175 ± 0.078^{AB}	1.223 ± 0.079^{A}
	2nd group (G_2)	1.010 ± 0.077^{aD}	1.054 ± 0.091^{abCD}	1.103 ± 0.07^{BCD}	1.177 ± 0.084^{AB}	1.206 ± 0.094^{AB}	1.232 ± 0.115^{A}
	3rd group (G_3)	0.986 ± 0.087^{aB}	1.131 ± 0.068^{abA}	1.134 ± 0.075^{A}	1.161 ± 0.072^{A}	1.191 ± 0.091^{A}	1.227 ± 0.107^{A}
	4th group (G_4)	1.076 ± 0.097^{a}	1.173 ± 0.076^{a}	1.133 ± 0.068	1.113 ± 0.056	1.112 ± 0.054	1.133 ± 0.068
Cortisol (µg/dL)	1st group (G_1)	20.663 ± 2.696^{aA}	22.86 ± 2.965^{aAB}	23.103 ± 2.819^{aB}	23.863 ± 2.965^{aBC}	24.663 ± 2.877^{aBC}	25.623 ± 2.926^{aC}
	2nd group (G_2)	15.42 ± 1.42^{b}	15.1 ± 1.38^{b}	14.42 ± 1.22^{b}	14.02 ± 1.096^{b}	14.26 ± 1.07^{b}	14.46 ± 1.24^{b}
	3rd group (G_3)	19.86 ± 1.898^{aB}	15.29 ± 1.27^{bA}	14.07 ± 1.18^{bA}	13.78 ± 1.15^{bA}	13.62 ± 1.12^{bA}	13.55 ± 1.05^{bA}
	4th group (G_4)	14.90 ± 1.2^{b}	13.7 ± 1.07^{b}	13.48 ± 0.74^{b}	13.26 ± 0.62^{b}	13.09 ± 0.67^{b}	12.77 ± 0.78^{b}
Prolactin (ng/mL)	1st group (G_1)	241.10 ± 22.07^{c}	236.40 ± 21.87^{c}	235.20 ± 21.65^{c}	238.05 ± 20.09^{c}	242.69 ± 21.44^{c}	247.15 ± 21.1^{c}
	2nd group (G_2)	310.90 ± 25.8^{ab}	316.70 ± 25.85^{b}	324.30 ± 24.16^{b}	331.16 ± 24.48^{b}	327.34 ± 25.62^{b}	331.35 ± 28.37^{b}
	3rd group (G_3)	286.6 ± 27.34^{bC}	313.88 ± 25.39^{bBC}	339.10 ± 22.33^{bAB}	347.11 ± 24.55^{bAB}	352.80 ± 25.58^{bAB}	360.54 ± 30.27^{bA}
	4th group (G_4)	326.940 ± 25.49^{aE}	372.50 ± 27.27^{aD}	406.97 ± 29.03^{aC}	434.30 ± 34.38^{aBC}	459.10 ± 39.47^{aAB}	474.62 ± 37.35^{aA}

Data are expressed as mean ± SE

[a, b, c] *Superscript: Mean significance difference among groups in the same column on P<0.05*

[A, B, C, D, E] *Superscript: Mean significance difference among administration times in the same row on P<0.05*

increase compared to the other two groups, G_2 and G_3. There was a non-significant change in T_4 levels during the experimental period between all groups. There was a gradually non-significant decrease in T_3 level of G_1 until the week 4. A significant (P<0.05) increase in the T_3 level of G_2, G_3, and G_4 from week 2 to week 5 compared to G_1. TSH levels results revealed a gradual increase in G_1, G_2, and G_3 from week 1 to week 5 with only a significant (P<0.05) decrease in G_1 on the 1st day post-AI compared to G_2, G_3, and G_4 and also a significant (P<0.05) decrease on the week 1 compared to G_4. There was a significant (P<0.05) increase in cortisol levels of G_1 throughout the experimental period compared to G_2, G_3, and G_4 groups. On the other hand, there was a significant (P<0.05) increase in the prolactin level of G_4 throughout the experimental period compared to G_1, G_2, and G_3. In the same time, there was a significant (P<0.05) increase in prolactin levels of G_2 and G_3 throughout the experimental period compared to G_1.

The results of oxidative stress biomarkers and antioxidants activities *(Table 4)* revealed a significant (P<0.05) increase in MDA and a significant (P<0.05) decrease in GSH, CAT, SOD, and TAC activities in G_1 compared with the other groups (G_2, G_3, and G_4) from week 1 to week 5 post-AI. At the same time, there was a significant (P<0.05) decrease in MDA and a significant (P<0.05) increase in GSH, CAT, SOD, and TAC of G_4 compared to the other two groups of vitamin E and selenium administration G_2 and G_3 on the most of the periods post-AI.

The results of pregnancy diagnosis *(Table 5)* revealed that the conception rates were 2/5 (40%), 3/5 (60%), 3/5 (60%), and 4/5 (80%) in G_1, G_2, G_3, and G_4, respectively.

Table 4. *Effects of vitamin E and selenium administration of ovsynch synchronized Friesian cows on serum oxidative stress biomarkers during hot season*

Parameters	Groups	Times					
		0-day	Week 1	Week 2	Week 3	Week 4	Week 5
MDA (nmol/mL)	1st group (G_1)	1.785±0.139^{a}	1.830±0.134^{a}	1.759±0.14^{a}	1.744±0.136^{a}	1.775±0.125^{a}	1.797±0.154^{a}
	2nd group (G_2)	1.461±0.128^{b}	1.414±0.108^{b}	1.360±0.093^{b}	1.395±0.097^{b}	1.417±0.099^{b}	1.431±0.091^{b}
	3rd group (G_3)	1.709±0.123^{aA}	1.394±0.107^{bB}	1.287±0.103^{bBC}	1.206±0.082^{cC}	1.163±0.103^{cC}	1.209±0.102^{cC}
	4th group (G_4)	1.354±0.128^{bA}	1.226±0.06^{cAB}	1.106±0.071^{cBC}	1.013±0.084^{dC}	0.982±0.055^{dC}	1.1040±0.078^{cC}
GSH (mg/dL)	1st group (G_1)	2.297±0.179^{c}	2.264±0.196^{d}	2.309±0.122^{c}	2.452±0.141^{d}	2.377±0.116^{d}	2.305±0.113^{d}
	2nd group (G_2)	4.129±0.362^{aC}	4.448±0.383^{bB}	4.553±0.384^{bA}	4.339±0.363^{cBC}	4.048±0.346 ^{CD}	3.818±0.277^{cD}
	3rd group (G_3)	2.900±0.176^{bC}	3.949±0.283^{cB}	4.837±0.326^{bA}	5.049±0.361^{bA}	4.998 ±0.358^{bA}	4.707±0.321^{bA}
	4th group (G_4)	4.254±0.324^{aA}	5.094±0.407^{aB}	5.522±0.438^{aC}	5.681±0.449^{aC}	5.767±0.433^{aC}	5.547±0.351^{aC}
CAT (U/mL)	1st group (G_1)	7.751±0.604^{b}	7.638±0.661^{c}	7.592±0.671^{c}	7.715±0.682^{d}	7.742±0.684^{d}	7.558±0.656^{d}
	2nd group (G_2)	13.529±1.189^{aAB}	14.178±1.218^{bAB}	15.084±1.26^{bA}	14.665±1.199^{cAB}	13.721±1.12^{cAB}	13.020±1.15^{cB}
	3rd group (G_3)	8.952±0.727^{bC}	13.845±1.192^{bB}	15.636±1.156^{bA}	16.79±1.314^{bA}	16.86±1.439^{bA}	16.216±1.462^{bA}
	4th group (G_4)	13.901±1.054^{aB}	17.539±1.277^{aA}	18.484±1.355^{aA}	18.719±1.478^{aA}	19.023±1.535^{aA}	18.865±1.423^{aA}
SOD (U/mL)	1st group (G_1)	13.422±1.046^{c}	13.228±1.144^{c}	13.494±0.714^{c}	14.331±0.826^{c}	13.891±0.681^{d}	13.47±0.66^{d}
	2nd group (G_2)	26.16±2.587^{aBC}	28.10±2.844^{bAB}	30.448±2.321^{bA}	30.134±2.34^{bA}	26.979±1.819^{cB}	24.074±1.718^{cC}
	3rd group (G_3)	16.893±1.401^{bC}	27.02±2.03^{bB}	31.63±2.373^{abA}	33.67±2.77^{aA}	32.06±2.18^{bA}	29.81±2.39^{bA}
	4th group (G_4)	27.77±2.92^{aC}	31.73±2.79^{aB}	34.22±2.66^{aAB}	35.36±2.87^{aA}	36.31±2.42^{aA}	34.63±2.35^{aAB}
TAC (mU/L)	1st group (G_1)	1.089±0.085^{b}	1.073±0.093^{c}	1.094±0.058^{c}	1.162±0.067^{c}	1.127±0.055^{d}	1.092±0.054^{d}
	2nd group (G_2)	1.34±0.089^{aB}	1.394±0.129^{bAB}	1.492±0.122^{bA}	1.496±0.114^{bA}	1.391±0.102^{cAB}	1.324±0.108^{cB}
	3rd group (G_3)	1.185±0.073^{bC}	1.357±0.097^{bB}	1.576±0.128^{bA}	1.636±0.134^{bA}	1.654±0.142^{bA}	1.573±0.126^{bA}
	4th group (G_4)	1.418±0.122^{aD}	1.531±0.13^{aCD}	1.632±0.129^{aBC}	1.719±0.143^{aAB}	1.762±0.152^{aA}	1.714±0.121^{aAB}

Data are expressed as mean ± SE

$^{a, b, c, \text{and } d}$ *Superscript: Mean significance difference among groups in the same column on P<0.05*

$^{A, B, C, \text{and } D}$ *Superscript: Mean significance difference among times of administration in the same row on P<0.05*

Table 5. *Effects of vitamin E and selenium administration of ovsynch synchronized Friesian cows on conception rate during hot season of each group (n=5)*

Parameter	Rate	Groups			
		1st Group (G_1)	2nd Group (G_2)	3rd Group (G_3)	4th Group (G_4)
Conception rate	Number	2	3	3	4
	Percent (%)	40	60	60	80

Discussion

In this study, ovsynch synchronization was provided using a GnRH and $PGF_2\alpha$ combination protocol, consistent with the previous studies in buffalo [21], and Friesian cows [22]. The results showed decreased follicular number and diameter in both G_1 and G_3 groups that did not receive vitamin E and Se supplementation. This decrease may be attributed to the adverse impact of heat stress during the hot season on reproductive performance and fertility [1,2]. Heat stress at the beginning of ovulation reduces the diameter and volume of the dominant follicle [23]. Additionally, it may directly impair the cellular function of reproductive cells, influence follicular maturation and waves [3], and result in smaller dominant follicles due to decreased feed intake during the hot season [5]. Heat stress adversely affects fertility by disrupting follicles and oocytes, likely due to oxidative damage [24]. Elevated temperatures in the hot season reduces feed intake that may compromise the energy balance of cattle and/or disrupting the hypothalamic-hypophyseal-ovarian axis. These factors impair the reproductive performance of the cow and compromise the quality of oocytes, and corpora lutea [25]. Vitamin E and selenium administration in group G_2 and G_4 led to increased follicular number and diameter, highlighting their

role in enhancing reproductive performance and follicular development [26,27]. Administration of vitamin E improves fertility in cows by regulating the free radicals within ovarian tissues. It was thought that this fertility improvement is likely attributed to the intracellular antioxidant activities of vitamin E and selenium which protect the cell membranes from oxidative damage by scavenging reactive oxygen radicals [28]. Notably, reactive oxygen species play important roles in ovulation, oocyte maturation, corpus luteum dynamics, implantation, and fetal development [29].

The hormonal profile revealed a gradual increase in progesterone concentration across all groups, especially after insemination which correlated with embryonic development [30]. The groups administered vitamin E and selenium showed a significantly higher progesterone concentration compared to group G1 (control group), consistent with previous research [31,32]. These results are likely related to the role of vitamin E and selenium in improving the resumption of ovarian activity by contributing to progesterone production by the corpus luteum [12].

Thyroid hormones revealed a gradual decline in T_3 levels alongside a corresponding elevation of TSH in group G_1 starting from the 1st week post-AI. These findings were consistent with previous studies in pregnant cows [33,34]. Reduced T_3 levels during the hot season could be attributed to the negative impact of elevated temperature on thyroid function and thyroid hormone levels [7]. This decrease in thyroid hormones may aid animals in acclimatizing to heat stress, as reduced thyroid concentration leads to decreased cellular oxygen consumption and metabolic heat production [35]. The significant increase of T_3 in the groups which received vitamin E and selenium was consistent with Shakirullah et al.[36] who reported enhanced thyroid hormone production in sheep exposed to combined vitamin E and Se supplementation during heat stress. Selenium is involved in the metabolism of thyroid hormones. The enzyme 5-iodothyronine de-iodinase is a seleno-dependent [37], so co-administration of vitamin E and selenium may promote T3 levels by facilitating the de-iodination of T4 into its active form T3.

The increased cortisol level in G_1 are in agreement with previous studies, which have reported increased serum cortisol levels during heat stress in the hot season [38]. This finding shows that the cows experience heat stress during such periods. The higher serum cortisol level may be attributed to thermal stress activating the hypothalamic-pituitary-adrenal cortical axis (HPA) leading to enhanced secretion of the stress hormone cortisol [7]. The reduction of cortisol levels observed in the groups administered vitamin E and selenium was consistent with previous studies in sheep [36], and dairy cows [7]. These results highlight the potential role of vitamin E and selenium in heat stress acclimation, acting as anti-stress factors.

The decrease of prolactin level observed in G_1 compared to the other administered groups may be attributed to the exposure of animals to heat stress, which alters the concentration of hormones such as thyroid hormones, cortisol, and prolactin. Hence, these hormone levels could be used as indicators of stress in animals [8]. Additionally, heat stress reduces feed intake, and also leads to blood flow redistribution, immune system depression and alterations in endocrine functions ultimately affecting the productivity and reproductive performance in cattle [6]. The significant increase in prolactin levels observed in groups administered vitamin E and selenium might be related to the role of selenium and vitamins in stimulating prolactin synthesis and secretion [39]. Current results provide the impact of vitamin E and selenium administration in stimulating prolactin secretion and improving milk yield even during the hot season [26,27].

Despite the adverse effects of heat stress on fertility, including follicle and oocyte disruption potentially induced by oxidative damage, it was essential to assess oxidative stress markers in this study. An increase in MDA activity, as well as decrease in GSH, CAT, SOD and TAC activities within G_1 compared to the administered groups, could be attributed to heat stress induced reduction in antioxidant activity, leading to oxidative stress [9,24,40,41]. Heat stress provides overproduction of free radicals and reactive oxygen species (ROS), disrupting the steady-state concentrations of free radicals, leading to both cellular and mitochondrial oxidative damage. Additionally, the decline in glutathione levels (glutathione insufficiency) is characteristic of reduced glutathione synthesis [42]. Decreased serum TAC concentration may be referred to thermal stress, suggesting that the antioxidant potential as a free radical scavenger has become depleted. Additionally, decreased SOD levels may reveal also endogenous antioxidant mobilization to neutralize free radicals [43]. Lipid peroxidation, mainly involving polyunsaturated fatty acids produces lipid peroxides, with MDA being the most prevalent among them [44]. Improving antioxidant activities in groups administered vitamin E and selenium were in accordance with previous studies in sheep [36], dairy cows [26], buffalos [45], which are in corroboration with the current findings. This condition indicates the positive effects of vitamin E and selenium, which have antioxidant roles. They are important components of the antioxidant defense system, contributing significantly to immune function and reproductive success through their involvement in essential enzymatic reactions [11]. Vitamin E acts as an intra-cellular antioxidant, and scavenging ROS thereby protecting cellular membranes from oxidative damage. Selenium acts as a co-factor in the glutathione

peroxidase enzyme system responsible for extracellular detoxification of free radicals [46].

Pregnancy diagnosis revealed an improved conception rate in groups administered vitamin E and selenium, consistent with findings from previous studies [26,32,47] reporting administration of vitamin E and selenium during late pregnancy improved conception rates in subsequent seasons. This provides the role of vitamin E and selenium to minimize postpartum disorders and improve cow's reproductive efficiency. Hemingway [13] who reported that supplementation of vitamin E plus selenium before mating improved the conception rate of cattle and sheep by decreasing early embryonic deaths. Also, administration of selenium and/or vitamin E combinations has been associated with increased pregnancy rates [14].

In conclusion, heat stress during hot season adversely affects reproductive performance by suppression of reproductive hormones production and enhancement of oxidative stress. Vitamin E and selenium administration reduces the effects of heat stress, acting as potent anti-oxidant activators, which involvement in crucial enzymatic reactions significantly contributes to reproductive success. Administering two doses of vitamin E and selenium, before synchronization and at artificial insemination, provides more impact on the conception rate compared to a single dose administered either before synchronization or pre artificial insemination.

Declarations

Availability of Data and Materials: The data sets analyzed during the current study are available from the corresponding author H. M. Yousif on reasonable request.

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Research Article

Effect of Propofol Induction on Antioxidant Defense System, Cytokines, and CD4+ and CD8+ T Cells in Cats

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Abstract

We investigated the effects of propofol on the antioxidant defense mechanisms, pro and anti-inflammatory cytokine expressions and specific defense processes in the study since these parameters play a significant role in postoperative complications, regulation of immune reactions, and wound healing. Twenty male cats were included in the study, anesthesia protocol was induced by IV administration of 6 mg/kg of propofol. Blood samples were harvested right before (T0) and fifteen minutes after (T1) propofol injection. Serum malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), IL-4, IL-8, TNF-α, IL-1β, and IFN-γ levels; the number of CD4+, CD8+ T cells and CD4/CD8 ratio in peripheral blood were determined. Propofol reduced the serum MDA and GSH-Px, while CAT and SOD levels remained unchanged. Furthermore, propofol did not impact serum IL-8, TNF-α, and IL-1β levels. Contrastingly, IFN-γ level tended to elevate, and serum IL-4 level was significantly increased. On the other hand, the CD8+ T cell population was significantly decreased, while the number of CD4+ T cells and the CD4/CD8 ratio were unaffected. Briefly, propofol did not adversely affect oxidative defense mechanisms, proinflammatory and anti-inflammatory cytokine cascade, and cell mediated immunity. Considering the insufficiency of cats' hepatic drug metabolism, we may conclude that propofol is a safe product regarding the investigated parameters.

Keywords: Antioxidant, Cat, Cytokine, Propofol, T cells

Introduction

Propofol (2,6-diisopropyl-phenol) is a sedative and hypnotic agent widely used to induce and maintain anesthesia in companion animals. It is favored in humans and animals due to the rapid induction of anesthesia, short action time, and fast elimination [1]. Propofol is mainly metabolized directly in the liver in humans, while, in cats, it is initially oxidized to 4-hydroxy propofol in the lungs and then eliminated due to the hepatic UDP-glucuronosyltransferase enzyme deficiency [2]. The relatively slow and inadequate extrahepatic metabolization of propofol impacts the cat's physiological status [3].

Oxidative stress is the impairment of the balance between antioxidants and free radicals in favor of the latter. It is reported that oxidative stress occurs because tissue perfusion and oxygenation changes during anesthesia [4]. In addition, feline erythrocytes are more prone to oxidative stress than other species due to relatively high concentrations of oxidizable sulfhydryl compounds. Particularly, repetitive anesthesia was indicated to have exerted oxidative damage in cats [3]. On the other hand, the antioxidant properties of propofol were shown in *in-vitro* and *in-vivo* studies conducted with humans and experimental animals [5]. Preoperative oxidative stress impacts postoperative wound healing, and its elevated levels play a role in potential complications. Therefore, considering the cats' susceptibility to oxidative stress, the potential effect of propofol on oxidative defense mechanisms is worth being investigated in this species.

Cytokines are small proteins that serve in inter-cellular signaling, regulating immune cells' development,

proliferation, function, and survival, and thus, they play a crucial role in inflammation and infections. Cytokines are rapidly released in response to stimuli and tissue injury due to cytokine-encoding genes' fast-acting transcription and translation processes [6]; thus, short-term applications, like anesthesia, are highly likely to affect cytokine production. Changes in cytokine expression or impaired balance between pro and anti-inflammatory cytokines impact the efficacy of immune response and wound healing, exerting potential postoperative complications [7]. The potential effects of anesthetics on cytokine production have been evaluated in humans and experimental animals [8]. In cats, cytokine levels have been investigated during infectious and noninfectious inflammation [9]. Studies emphasizing the need for further exploration in cats solely focus on cytokine production during anesthesia.

This addition emphasizes the need for further research on cytokine production specifically during inflammation in cats. The function of T cells is highly significant since they are the fundamentals of the specific defense system, inducing both cytotoxic and antigen-producing activities benefiting the defense responses [10]. These cells are also closely associated with cytokines, since it is through cytokines that helper T cells are able to recognize the invading pathogen and present it to the specific defense system [11]. Therefore, cytokines and T cells should be simultaneously assessed while investigating the potential effects of anesthetics. The relevant connection was relatively investigated in humans [12]; however, few documented studies address the animal phenomenon [13]. Therefore, examining the effects of propofol anesthesia on cellular immune responses in cats would be beneficial.

The dispersion of propofol into the tissues and its metabolism and elimination is distinct in cats compared to dogs and other species [14]. This difference has the potential to alter the effects of anesthetics on the physiological system. However, more data are required concerning the issue in question. Therefore, this study was designed to reveal the potential effects of propofol on the cardiopulmonary system, antioxidant defense mechanisms, pro and anti-inflammatory cytokine expressions, and specific defense processes since all these parameters play a significant role in potential postoperative complications, regulation of immune reactions, and wound healing.

Material and Methods

Ethical Statement

This study was carried out with the permission of Istanbul University Local Ethics Committee (Approval no: 35980450-050.01.04). Additionally, informed consent forms were obtained from the owners.

Animals

Twenty cats that had to be operated on for various conditions were included in the study. The cats comprised 1-6-year-old males to rule out age and gender-associated variation *(Table 1)*. Of the cases in the study, 8 were castration, 2 were tooth extraction, 2 were blepharoplasty, 1 was urethrostomy, 1 was cystotomy, 1 was tibial fracture osteosynthesis, 1 was wound revision, 1 was removal of osteochondrodysplastic exostoses, 1 was femoral fracture osteosynthesis, 1 patient with inguinal cryptorchid, and 1 patient with enucleation underwent anesthesia.

Anesthesia

According to the American Society of Anesthesiologists (ASA) physical status classification system, ASA I and II patients were subjected to evaluations. All patients preoperatively underwent a routine physical exam, including hemograms and biochemical testing. Food and water intake ceased 6 h and 1 h before the induction of anesthesia, respectively. Each cat was administered IV 6 mg/kg of propofol through a 22 G angiocath (Vasofix; B. Braun Melsungen AG, Germany) inserted into the v. cephalica antebrachii.

Heart rate (HR), respiratory rate (fR), end-tidal CO_2 ($ETCO_2$), oxygen saturation (SpO_2), and body temperature were recorded before and during anesthesia. The heart

Table 1. *Demographic data of the cats included in the study*

Breed	n	Age (year)		Body Weight (kg)	
		Mean	SD	Mean	SD
Mixed breed	10	2.45	1.34	4.15	0.98
Scottish Fold	4	2.12	0.47	4.67	1.01
Exotic Shorthair	2	2	1.41	3.5	0.7
Persian cat	2	1.5	0.71	2.75	0.35
Siamese	1	4		4	
Norwegian forest cat	1	4		7	
Total	20	2.4		4.13	1.2

rhythm was monitored by a multifunctional ECG monitor (Advisor V9212 AR; Surgivet, Waukesha, WI, USA) based on lead II. The respiratory rate was initially determined by monitoring the chest movement and then the movements of the reservoir bag after endotracheal intubation.

The $ETCO_2$ levels were recorded by a capnometer integrated into the ECG monitor (Advisor V9212 AR; Surgivet, Waukesha, WI, USA), attached to the intubation tube. The SpO_2 was measured on the tongue mucosa using a pulse oximeter probe (Advisor V9212 AR; Surgivet, Waukesha, WI, USA). The body temperature was rectally taken by a digital thermometer (Omron, The Netherlands) during the anesthesia. Once the jaw muscles relaxed, endotracheal intubation was performed using appropriate-sized intubation tubes (Rüsch, Germany). General anesthesia was initiated by 4% and maintained by 2% isoflurane, after the blood draw at the 15th min following propofol induction (Royal-77 Anesthesia Machine CE 0434, Seul KOREA).

Blood Sampling

Blood samples were harvested through v. jugularis right before (T0) and 15 min after propofol administration prior to surgical incision (T1) and collected into anticoagulant and non-anticoagulant tubes for hemogram plus flow cytometry and to obtain sera stored at -80°C for further analyses, respectively.

Determining the Effect of Propofol on Antioxidant Levels

Serum malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels were measured to assess the potential effect of propofol on oxidative defense mechanisms by the ELISA method using commercial kits specific to the cat (Abbkine Scientific, Hubei, China) according to the manufacturer's instructions.

Estimating the Effect of Propofol on Cytokine Expression Levels

Proinflammatory IL-8, IL-1β, TNF-α, and IFN and anti-inflammatory cytokines IL-4 were measured by the ELISA method using commercial kits specific to the cat (Abbkine Scientific, Hubei, China) according to the manufacturer's instructions. Briefly the stock solution (containing 400 ng/L cytokine) included in the kit was subjected to a series of dilutions by the sample diluent to obtain the standard solutions. In total, 150 µL of the former diluted solution was transferred to the latter in each dilution step, ensuring the final standard solutions contained a maximum assay concentration of 200 ng/L and minimum of 12.5 ng/L. Serum samples (50 µL) processed with the standard solutions were subjected to ELISA analysis according to the manufacturer's instructions. Absorbance measurements were taken at a wavelength of 405 nm using an RT6000 spectrophotometer (Rayto, Shenzhen, China). Intra- and inter-assay variabilities were determined to be <9% and <11%, respectively, based on information provided by the manufacturer.

Flow Cytometry Analysis

Cat-specific monoclonal CD3+ (CD3-FITC), CD4+ (CD4-PE), and CD8+ (CD8α-PE) antibodies (Southern Biotech© Birmingham, USA) were used to label T cells. Before the analyses, CD3-FITC and CD4-PE were diluted at a ratio of 1:50 and CD8α-PE at a ratio of 1:100 using PBS and stored at 2-8°C. Two flow cytometry tubes (12x75 mm, polystyrene) were prepared for each specimen, and 10 µL of CD3 FITC + CD4 PE and CD3 FITC + CD8 PE were pipetted into the tubes, respectively, and then 100 µL of whole blood was added into each tube. The tubes were vortexed at medium speed for 2 sec and left for 15 min-incubation in the dark, enabling the leukocytes to be stained with the antibodies. For removing the erythrocytes, 2 mL of the lysing solution was pipetted into each tube, which was vortexed and left to dark incubation for 10 min, centrifugated at 300 g for 5 min. The obtained supernatant was disposed of by a pipette. Then, 2 mL of PBS was added into the tubes, which were centrifuged again following the same procedure, and the supernatant was pipetted out. Finally, 500 µL of PBS was added into the tubes, and the specimens containing rinsed and monoclonal antibody-labeled leukocytes were read on the flow cytometer. The CellQuest program (Becton Dickinson, New Jersey. USA) was used for cytometric analysis. Twenty thousand cells per specimen were run through the flow cytometer, and CD4+ and CD8+ T cells were assessed by FL-1 and FL-2 detectors.

Statistical Analysis

The IBM SPSS Statistics Version 21 program (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The Shapiro-Wilk test was applied to determine whether or not the data were normally distributed. The difference between the parameters regarding T0 (before anesthesia) and T1 (after anesthesia) and the normally distributed data of the paired specimens were analyzed by the dependent t-test. Non-normally distributed data were analyzed by the Wilcoxon Signed Rank test. The results were presented as mean ± standard error of mean (SEM) for parametric and median and inter-quarterly range (IQR) for non-parametric data. The significance was estimated at a level of $P<0.05$.

Results

The effects of propofol on the cardiopulmonary system and body temperature is presented in *Fig.1*.

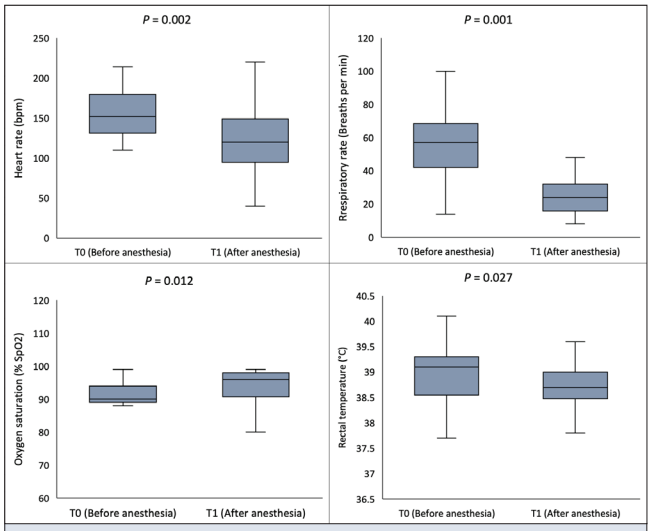

Fig 1. The effects of propofol on the cardiopulmonary system and body temperature. Data are presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values (n=20). Different letters above the columns indicate a significant difference between the groups, P<0.05 (n=20)

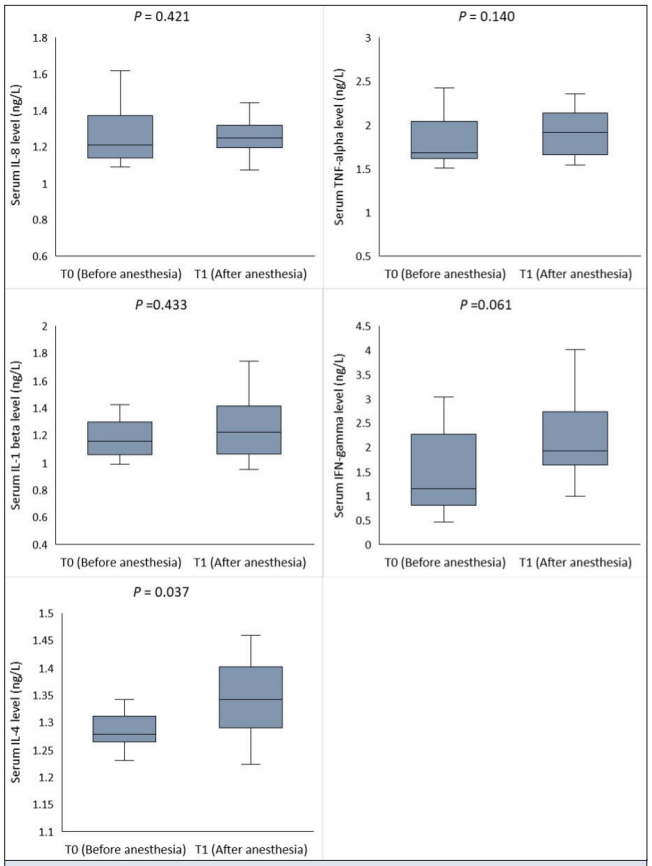

Fig 3. The effects of propofol on proinflammatory cytokines. Serum interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1B), interferon gamma (IFN-G), and interleukin 4 (IL-4) levels before sedation (T0) and 15 min after anesthesia induction (T1) in cats anesthetized with propofol. Data are presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values. Different letters above the columns indicate a significant difference between the groups, P<0.05 (n=20)

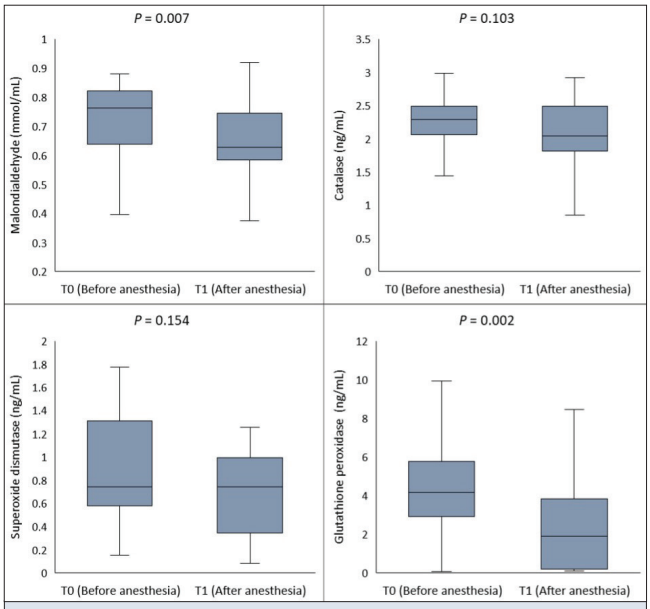

Fig 2. The effects of propofol on antioxidant defense responses. Serum malondialdehide (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) levels measured before sedation (T0) and 15 min after anesthesia induction (T1) in cats anesthetized with propofol. Data are presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values. Different letters above the columns indicate a significant difference between the groups, P<0.05 (n=20)

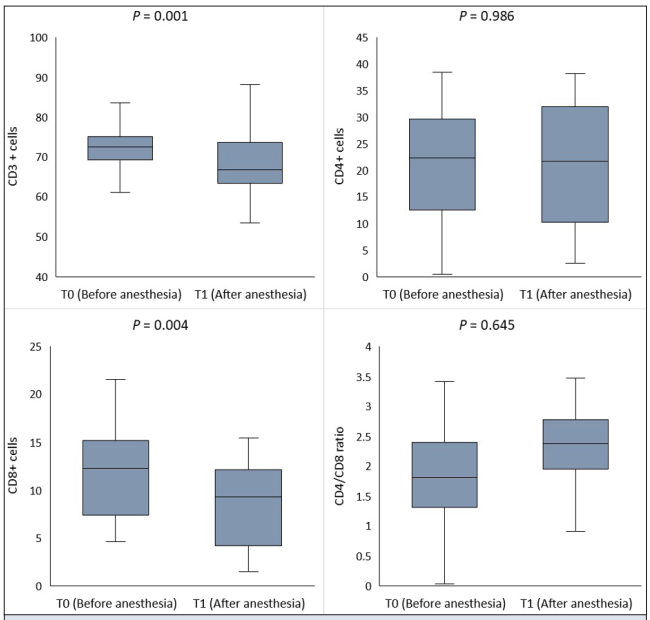

Fig 4. The effects of propofol on CD3+, CD4+, and CD8+ T cells. Peripheral blood CD3+, CD4+, CD8+ cells percentages and CD4/CD8 cell ratio before sedation (T0) and 15 min after anesthesia induction (T1) in cats anesthetized with propofol. Data are presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values. Different letters above the columns indicate a significant difference between the groups, P<0.05 (n=20)

The effects of propofol on antioxidant defense responses is presented in *Fig.2*. Serum MDA level was significantly decreased after propofol administration (P=0.007).

Likewise, serum GSH-Px level was also reduced (P=0.002). On the other hand, propofol did not affect serum CAT and SOD activities (P=0.103 and P=0.154, respectively).

The effects of propofol on proinflammatory cytokines is demonstrated in *Fig.3*. The data revealed no significant changes in serum IL-8, TNF-α, and IL-1β levels (P=0.421; P=0.140 and P=0.433, respectively). On the other hand, serum IFN-γ level was noted to have been increasing (P=0.061). Serum IL-4 level was significantly elevated after propofol anesthesia (P=0.037).

The effects of propofol on CD3+, CD4+, and CD8+ T cells is presented in *Fig.4*. The data revealed a significant decrease in CD3+ and CD8+ T cells (P=0.001 and P=0.004, respectively). In contrast, propofol anesthesia did not impact the number of CD4+ T cells and the CD4/CD8 ratio.

Discussion

Propofol leads to a dose-dependent decrease in systemic blood pressure, myocardial contractility, and cardiac output. Likewise, it was previously reported to have decreased the heart rate in rabbits [15]. On the other hand, documented studies indicated that dogs' heart rates remained unaffected [16]. In this study, unlike the dog, propofol anesthesia markedly decreased the heart rate in cats. Cats do not possess the UDP-glucuronosyltransferase enzymes found in humans and dogs, and they also lack conjugation enzymes such as N-acetyltransferase and thiopurine methyltransferase. The lack of these enzymes delays propofol's elimination [14], which was associated with a decreased heart rate in cats after propofol anesthesia.

IV administration of 6 mg/kg of propofol reduced the respiratory rate, and elevated the oxygen saturation in this study. In a previous study, the respiratory rate was similarly decreased, while, contrastingly, oxygen saturation remained unchanged in cats [17]. The increase in the SpO_2 level in our study was associated with the cats' intubation, followed by ventilation with 100% oxygen. A similar condition was previously reported in dogs anesthetized with propofol [16].

Hypothermia is a widely encountered complication of general anesthesia in small animal practice. In this study, hypothermia did not occur after propofol induction, yet, the body temperature was prominently decreased *(Fig.1)*. It was indicated that the body temperature was decreased by approximately 1.5°C in a short while in humans anesthetized with propofol [18]. Mild hypothermia [19] and unchanged body temperature [16], previously reported in dogs, were associated with the anesthetic dose.

Even though cats are quite susceptible to oxidative stress, few studies investigated this subject [20]. In this context, it is crucial to investigate the impact of propofol anesthesia on oxidative mechanisms. The MDA level is one of the biomarkers indicating oxidative stress in humans and animals, and an elevation in the MDA level points out oxidative stress. In previous studies, propofol anesthesia did not impact serum MDA levels in dogs [21]. On the other hand, propofol was reported to have reduced MDA levels in humans [22]. Our data revealed that propofol prominently reduced serum MDA levels, thus preventing oxidative stress. It is unlikely to elucidate the underlying mechanism thoroughly; nevertheless, propofol chemically resembles certain antioxidants such as α-tocopherol, butylated hydroxytoluene, and butylated hydroxyanisole. It was suggested that propofol neutralizes free radicals like the relevant antioxidants due to their chemical similarity [23], which was associated with our study's decrease in the MDA levels.

In the study, it was an intriguing finding that GSH-Px levels were decreased despite no increase in the MDA level, which raises the question of why the GSH-Px level decreased even though oxidative stress did not occur. A similar finding was previously demonstrated in humans, indicating that propofol reduced GSH-Px activity in the platelets by 28.3% [24]. Extensive oxygen exposition increases mitochondrial ROS production, and 2% of the oxygen entering the mitochondria emerges as ROS [25]. In the study, oxygen saturation was significantly elevated due to oxygen application through intubation, which was considered to be associated with the over-production of reactive oxygen molecules in the mitochondria, depleting the GSH-Px enzyme participating in the elimination of oxidative radicals. It was previously reported that the GSH-Px enzyme is abundant in the mitochondria and plays a significant role in eliminating the ROS that emerged [26]. Conclusively, the decrease in GSH-Px level was not associated with propofol but with the oxygen source administered.

The main function of superoxide dismutase (SOD) is to accelerate the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen [27]. In our study, the serum SOD level remained unchanged in the cats anesthetized with propofol. No data are available revealing the effect of propofol anesthesia on the serum SOD level, hampering the comparison of our findings. Nevertheless, some documented studies previously indicated that the serum SOD level was not impacted either in dogs [16].

It was noted in the study that propofol did not affect proinflammatory cytokines other than IFN-γ, of which level tended to elevate after anesthesia *(Fig.3)*. It was reported in humans that, after propofol induction, when the cells in the whole blood cultures were stimulated by lipopolysaccharides (LPS), a significant increase was recorded in IFN-γ secretion [8]. Similarly, propofol

increased IFN-γ production in lymphocyte [12], macrophage, and natural killer cell cultures [28]. However, unlike the mentioned *in-vitro* studies, propofol revealed a decrease in IFN-γ production in dogs [16]. Therefore, *in-vitro* studies must be supported by in-vivo research. The issue is more critical in cats since immune cellular or inflammatory responses differ from those of other species [29]. In this study, the data from previous *in-vitro* studies conducted with humans were clinically verified in cats. IFN-γ is crucial in coordinating non-specific immune processes, antigen presentation to the specific defense system, and lymphocyte-endothelial cell interactions [30]. IFN-γ is one of the major stimulants activating inactive macrophages [31]. Furthermore, it increased the cellular proliferation and maturation in the injured tissues of mice [32]. *In-vitro* studies showed that IFN-γ accelerated wound healing by supporting the injured tissues' repair in the defective areas [33]. Therefore, we consider that the IFN-γ level's tendency to increase during anesthesia might efficiently prevent postoperative infections and improve wound healing.

Propofol was shown to exert anti-inflammatory effects in humans [34], which was previously associated with the inhibition of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 [7]. Likewise, it was demonstrated that propofol inhibited TNF-α production in the type-II alveolar epithelial cells [35]. We may deduce that propofol also elicited an anti-inflammatory effect in cats. However, we assume propofol revealed such an effect not by inhibiting proinflammatory cytokines such as TNF-α or IL-1β but by increasing the anti-inflammatory cytokine IL-4's secretion.

The main source of IL-4 is T helper 2 (Th2) cells, a subtype of helper T cells [36]. When stimulated by various factors, helper T cells differentiate into Th1 and Th2 cells [37]. The transformation of T cells into Th2 and, thus, IL-4 secretion is inhibited by the leukocyte function-associated antigen-1 (LFA-1) [38]. Propofol has been shown to inhibit LFA-1 in humans [39]. Therefore, it was hypothesized that propofol may have increased IL-4 secretion in cats by abolishing LFA-1's inhibition on Th2 cells. Additionally, the inhibition of LFA-1 by propofol might also explain the tendency for increased production of IFN-γ. This is because Th1 cells, which are inhibited by LFA-1, are one of the main sources of IFN-γ [40]. Propofol's annihilation of the inhibitory activity of these cells might have increased the IFN-γ production.

Besides IL-4's inhibitory effect on proinflammatory cytokine expressions, it stimulates B cell activation and proliferation, which plays a role in antibody-mediated immune response [41]. The increase in the IL-4 level after propofol anesthesia was considered an affirmative output due to the restriction in potential postoperative infection and stimulation of antibody-mediated humoral immunity against potential risk for contamination.

Anesthetic agents are known to cause immunosuppression [42] and affect the subtypes of T lymphocytes [43]. In this study, no changes were recorded in CD4+ T cells and CD4+/CD8+ ratio; however, a significant decrease was detected in CD8+ T cells *(Fig. 4)*. Similarly, propofol induction in humans with lung cancer decreased CD8+ T cells, while CD4+ T cells remained unchanged [44]. No change was noted in the CD4+/CD8+ ratio in the patients with mammary cancer [45], which was compatible with our findings. It was reported in dogs that CD4+ and CD8+ T cells in the peripheral blood were decreased within a short time after propofol induction, yet they returned to their preoperative status after a while [13]. In the same study, the CD4+/CD8+ ratio remained unchanged, which was compatible with our findings.

CD8+ T cells are freely distributed between the blood circulatory system and the secondary lymphoid organs under homeostasis [46]. Expression of specific molecules on T and endothelial cells changes during infection or inflammation due to simultaneous effects of cytokines expressed and the antigenic agents, which impacts the CD8+ T cells' transfer from blood vessels to the tissues and from the tissues to the lymphoid organs and lymphoid vessels [47]. In this study, the decrease in CD8+ T cells in the peripheral blood vessels after propofol anesthesia might be associated with these cells' increased migration to the tissues since the cardinal molecule affecting the CD8+ T's transition from the blood vessels into the tissues is L-selectin expressed on the endothelial cells [48]. Propofol, along with remifentanil, is known to induce L-selectin expression [49]. Furthermore, in our study, the IFN-γ level's tendency to elevate after propofol induction might have increased CD8+ T cells' transfer to the tissues. It was also previously reported in an experimental model that when the IFN-γ-knockout mice received recombinant IFN-γ, vascular cell adhesion molecule-1 (VCAM-1) expression on cerebral blood vessels was increased, which stimulated the CD8+ T cells' transition into the tissues [50].

Anesthetic agents such as fentanyl, thiopental, and isoflurane were reported to have increased CD8+ T cells in the peripheral blood [43], suggesting that, unlike propofol, these anesthetics did not stimulate the mechanisms mediating cell migration from the blood vessels to the tissues. Regardless of the underlying mechanism, the decrease in the ratio of circulatory CD8+ T cells due to their transfer to the tissues is not considered an adverse event since naïve CD8+ T cells transferred to the tissues gain cytotoxic activity to directly demolish pathogen-infected cells and play a role in inflammatory cytokine production in the infection zone [47]. Therefore, they may offer drastic contributions to the host's defense responses in the postoperative period.

This study has some limitation. It was intended to evaluate the effects of propofol alone, without the effect

of isoflurane. Since we had to intubate the patient 15 min after giving a bolus dose of propofol, we were able to evaluate the effects of propofol after 15 min. In further studies, the effects of continuous infusion of propofol on cytokines and T cells can be evaluated.

The data obtained in the study revealed the suppressive effect of propofol anesthesia on the cardiorespiratory system in cats, which necessitates monitoring patients with heart conditions. Propofol also reduces body temperature; therefore, precautions should be taken against the potential risk of hypothermia during the surgery. Propofol did not adversely affect the antioxidant enzymes; on the contrary, it exhibited anti-inflammatory properties by reducing the MDA level and increasing IL-4 secretion. Furthermore, it did not negatively impact the cells of the specific defense system in the peripheral circulation.

In conclusion, propofol may enhances postoperative wound healing, reduces potential infection, and supports immune defense responses since it does not adversely impact the oxidative defense system, inflammatory/anti-inflammatory mechanisms, and cells of the specific defense system. Considering the cats' unique hepatic drug metabolism, propofol might be safely administered in this species regarding the investigated parameters.

Declarations

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

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Research Article

Correlation Between Body Measurements of *Gazella marica* Fawns After Weaning and Estimating Body Weight From These Measurements

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Abstract

This study aims to find out for the first time the change in body measurements of gazelle fawns from birth to weaning and the estimation of live weight based on body measurements (BW, BL, CC, CD, AC, SH) of a total of 42 fawns, 17 female and 25 male, taken after birth, and a total of 30 fawns, 15 female and 15 male, were taken after weaning. Weighings were made with electronic scales. Measurements were made with a 150 cm strip measure. At the end of the 165-day suckling period, female fawns gained an average live weight of 8.07 kg and male fawns gained an average live weight of 9.17 kg. There is no statistical difference between the measurements at birth and the body measurements after weaning depending on gender (P>0.05). In general, the highest correlation (0.910) according to body measurements is between chest circumference and body weight (P<0.01). In male fawns, body weight can be predicted by chest circumference at a rate of 89% (P<0.01). In female fawns body weight can be predicted by shoulder height and chest circumference at rate of 79% (P<0.01). By selecting breeding individuals based on the high correlations and prediction values investigated in this study we can ensure faster genetic progress in a gazelle herd.

Keywords: *Gazella marica*, Correlations, Estimating body weight, Body measurements, Body weight, Weaning

Introduction

Gazelles belong to the genus Gazella, from the antelope subfamily of the horned family, and have a wide habitat extending from China to North Africa. The lower range of gazelles, which belong to the *Gazella marica* species, extends from Yemen and Oman to Şanlıurfa [1-4]. Their numbers in the wild are reported to be 1750-2150 [5]. It is known that there are around 400-500 in the wild in Türkiye. The mating season begins in November and December and pregnancies last for about 5 months. Births generally occur from late April to mid-May. After a gestation period of five months, females usually give birth to one or two fawns [6]. Gazelle fawns suck their mother for approximately 4 months [7]. While suckling is most frequently observed in May, a few short-term suckling have been observed at least in August and in September [8]. However, the suckling period in small ruminants is kept shorter. For example the lactation period in lambs lasts approximately 3 months for economically. They are weaned from the age of 3 months and subjected to various fattening programs [9,10]. However, the main food source of gazelles in the first three months is milk. In addition to decreasing suckling behavior, grazing behavior is also increasing. Gazelles show interest in grass and exhibit grazing behavior when they are approximately 3-4 days old after birth [11]. Since gazelles grow rapidly, grazing behavior occurs inversely proportional to sucking behavior. As suckling decreases day by day, grazing also increases [8]. This plays an important role in the survival of baby gazelles. Birth weight has a major impact on the survival of offspring in ungulate species. Offspring with high birth rates have higher survival rates [12]. Male gazelle fawns are born heavier on average than female gazelle fawns. Additionally, single fawns have higher average birth weights than twin fawns [13,14].

Body weight and measurements are an important indicator of growth. These indicators depend on genetic and environmental factors [15]. It has also been reported that in *Capreolus capreolus*, male body size and higher horns are more effective for selection in males [16]. It has been reported livestock weight in lambs depends on breed, gender, nutrition, season, age, etc. [17]. Studies made on domestic and wild animals have reported positive correlations between body weight and body measurements [13,18,19]. Moreover, body weight can be estimated from body

measurements [20]. In a study made on goats, estimating live weight by chest circumference gives 91% confidence [21]. Many studies have been done on adult and young body measurements of gazelles [13,14]. In addition, the weights of their offspring were determined by the previous study [13]. To what extent the change in body measurements occurs during the suckling period has been wondered.

Based on this information, it was aimed to reveal to what extent the body measurements of gazelle fawns, whose body measurements were recorded after birth, changed after they were weaned naturally. We also aimed to find the unknown body measurements of gazelle fawns and to find the change in body measurements during the suckling period. In addition, we tried to find out whether there are differences in body measurements by gender and whether there is a correlation between body weight and body measurements, both on a gender basis and in general. We also tried to find out whether body weight could be predicted from body measurements.

Following the results, we aimed to be obtained, to lead the formation of weaning programs for gazelle fawns for gazelle breeding stations, the number of which is increasing day by day in our country and around the world. We also aimed to increase the importance of records made during the lactation period for the selection of gazelle fawns according to their phenotypic characteristics.

Material and Methods

Ethical Statement

The research permissions were obtained with the letter dated 23.03.2023 and numbered E-21264211-288.04-9308521 from the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks.

Ethical approval was also obtained by Harran University Animal Experiments Local Ethics Committee Presidency with decision number 2023/01/02 and ethics committee permission dated 11/01/2023 and numbered 196981.

Animals

The research was carried out at the Ceylan Breeding Station in Yeniköy (Cudeyde) of Haliliye District of Şanlıurfa Province. The origin of gazelles in Şanlıurfa Ceylanpınar Gazelle Breeding Station. The location of the Breeding Station is at 37° 07′ 31″ North latitude and 38° 50′ 24″ East longitude and is located at an altitude of 449 meters above sea level.

Study Program

Fawns were recorded by taking measurements at 1-2 days of age from birth and after weaning. The study started on May 1 and continued until November 30. Weaning time

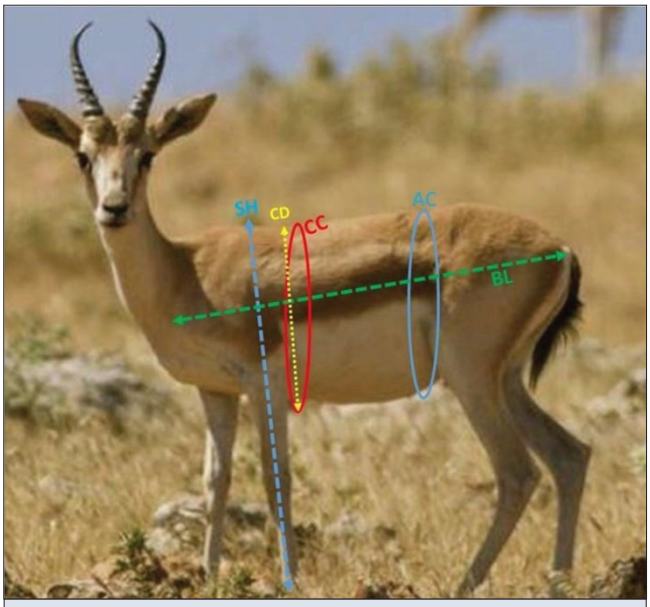

Fig 1. Body measurements *Gazella marica*. BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth

was recorded as 165 days. Because the suckling detected was observed during this period. In addition, after this period, pre-mating preparations of the broodstock begin. The fawns born on May 15 were weaned in November. Since estrus begins in October and November, suckling is completed at the end of this period. In addition, this period was kept optimal as it allowed mothers' mammary glands to rest for the next pregnancy and birth. Body measurements taken are as follows: Body Weight, (BW), Body Length, (BL), Chest Circumference, (CC), Chest Depth, (CD), Abdominal Circumference, (AC), Shoulder Heigth (SH) *(Fig. 1)* [30]. After birth, body measurements were taken from 25 male and 17 female fawns. All of these individuals were born as a result of a single-fawn birth. The ages of the mothers ranged from 2 to 8 years. However, after 165 days due to deaths of male and female offspring and the sale of male offspring, final body measurements were taken only from 15 males and 15 females. While the herd was divided into groups after weaning and before the mating of the mothers, these body measurements of the fawns were taken again and recorded. Adult and young males were kept separate from the herd during pregnancy and birth to prevent harm to the mother and the fawns. It is kept together with females only during the mating season.

Care and Feeding of Mother and Fawns

Gazella fawns suckled their mothers freely. The fawns stayed with their mothers throughout the lactation period. No artificial milk or hand feeding was used. While the fawns started grazing, they consumed the *Medicago sativa* grown in the area. They consumed

ready-made fattening feed ration as supplementary feed. The content of the ready feed given was as follows: 18% Crude protein, 10% Crude fiber, 3% Crude fat, Dry matter 88%, Metabolic energy 2800 Kcal/kg. They were fed on a total of 10 decares of land, 8 decares of grazing land and 2 decares of empty land. *Medicago sativa* is cultivated in the 8-decare cultivated area for 12 months of the year and is constantly irrigated. The 8-decare area is divided into two according to need, and grazing is done alternately. Mother gazelles were given cracked barley (Crude protein 10.30%, Crude fat 2.48%, Dry matter 92.56%, NDF 20.7%) as concentrated feed as well as dairy feed. There was 120 gazellas in breeding stations. When we were starting to feding, barley and dairy feed was given to 120 gazellas 3 times a day, with a total of 45 kg of cracked barley, 13.5 kg per day.

Weighing and Measurements

Portable Electronic Scale PLT Power A08 model scale, which can weigh up to 50 kg, has a sensitivity of 5 grams for 0-10 kg and a sensitivity of 10 grams for weights of 10-50 kg, was used for weighing the fawns. Additionally, another TEM BM2CAA 35*40 150 kg electronic scale was used to control the weighing results. A 150 cm strip measure was used for body measurements. Body measurements; Body Weight, (BW), Body Length, (BL), Chest Circumference, (CC), Chest Depth, (CD), Abdominal Circumference, (AC), Shoulder Heigth (SH), postnatal and post-weaning measurements were recorded.

Vaccination, Protection and Treatment

As a vaccine, a combination vaccine is given twice a year only against enterotoxemia. Vitamin B, A, D, E and K were used as vitamin supplement. Mineral licking stone blocks were used for salt and minerals.

Statistical Analysis

Normality test of body measurements was made by Shapiro-Wilk. The data are normally distributed according to Shapiro-Wilke. According to the relation between genders and body measurements was tested with Independent Samples T test. We analyzed correlation body weight with body measurements with Pearson Correlation test. We performed Linear Regression analysis to estimate body weight by body measurements. All 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 coefficient (R2) and the residual standard deviation (RSD).

Significance levels were taken as $P<0.05$. All data were analyzed by SPSS (version 28.0) statistical program.

Results

The average birth weight of male fawns was found as 1.71 kg and the average birth weight of female fawns was 1.64 kg. The general average body weight was found to be 1.68 kg. The average body length of female fawns is slightly longer than that of male fawns. CD and SH were slightly higher in male offspring than in female fawns, but CC and AC were on average higher in female fawns than in males. There are no significant differences between genders and body measurements ($P>0.05$) *(Table 1)*.

Table 1. *Body measurements of birt*

Measure		Females (n = 17)		Males (n = 25)		P
		Min–max	Mean±SE	Min–max	Mean±SE	
General (G.)	BW (kg)	0.91-2.30	1.64±0.08	1.26-2.36	1.71±0.06	0.47
	BW (kg) G.		1.68±0.51	0.91-2.36		
	BL (cm)	16.0-29.0	23.77±0.73	20.5-29.0	23.44±0.43	0.69
	BL (cm) G.		23.57±0.39	16.0-29.0		
	CC (cm)	21.0-30.5	26.28±0.57	22.0-30.0	26.14±0.43	0.84
	CC (cm) G.		26.20±0.34	21.0-30.5		
	CD (cm)	7.0-11.0	9.15±0.28	7.5-11.0	9.20±0.22	0.88
	CD (cm) G.		9.18±0.17	7.0-11.0		
	AC (cm)	21.0-33.0	26.38±0.71	21.5-30.0	25.76±0.45	0.44
	AC (cm) G.		26.01±0.39	21.0-33.0		
	SH (cm)	25.0-35.0	30.29±0.62	28.0-38.0	31.74±0.55	0.09
	SH (cm) G.		31.16±0.42	25.0-38.0		

BW: Body Weight, BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth, n: Number of induviduals, SE: Standart Error, Min: Minimum, Max: Maximum

Table 2. *Body measurements after weaning*

Measure		Females (n = 15)		Males (n = 15)		P
		Min-Max	Mean±SE	Min-Max	Mean±SE	
General (G.)	BW (kg)	8.33-11.58	9.71±0.27	6.23-14.44	10.90±0.66	0.11
	BW (kg) G.		10.31±0.37	6.23-14.44		
	BL (cm)	42.0-56.0	48.53±1.04	39.0-57.0	50.70±1.41	0.23
	BL (cm) G.		49.62±0.89	39.0-57.0		
	CC (cm)	47.5-55.0	52.03±0.61	44.5-60.0	53.10±1.21	0.44
	CC (cm) G.		52.57±0.6	44.5-60.0		
	CD (cm)	17.5-23.0	20.70±0.40	15.0-26.0	21.47±0.79	0.39
	CD (cm) G.		21.08±0.44	15.0-26.0		
	AC (cm)	41.0-57.0	49.63±1.24	42.0-57.0	50.43±1.10	0.63
	AC (cm) G.		50.03±0.82	41.0-57.0		
	SH (cm)	45.0-53.0	49.70±0.58	46.0-56.0	51.53±0.77	0.07
	SH (cm) G.		50.61±0.50	45.0-56.0		

BW: Body Weight, BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth, n: number of induviduals, SE: Standart Error, Min: Minimum, Max: Maximum

Table 3. *Body measurements changes over 165 days*

Measure		Females (n = 15)		Males (n = 15)		P
		Min-Max	Mean±SE	Min–max	Mean±SE	
General (G.)	BW (kg)	6.70-9.58	8.07±0.25	4.87-12.43	9.17±0.60	0.10
	BW (kg) G.		8.61±0.34	4.87-12.43		
	BL (cm)	17.0-32.5	24.60±1.33	14.0-35.0	27.50±1.34	0.14
	BL (cm) G.		26.05±0.97	14.0-35.0		
	CC (cm)	21.0-29.5	25.71±0.68	19.5-32.0	26.93±0.96	0.31
	CC (cm) G.		26.32±0.59	19.5-32.0		
	CD (cm)	9.0-14.0	11.60±0.39	7.0-17.0	12.13±0.74	0.53
	CD (cm) G.		11.87±0.41	7.0-17.0		
	AC (cm)	14.5-32.0	23.07±1.43	19.5-31.0	24.87±0.72	0.27
	AC (cm) G.		23.97±0.80	14.5-32.0		
	SH (cm)	15.0-24.0	19.37±0.69	16.5-24.0	20.20±0.56	0.36
	SH (cm) G.		19.78±0.45	15.0-24.0		

BW: Body Weight, BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth n: Number of induviduals, SE: Standart Error, Min: Minimum, Max: Maximum

The body measurements of the same fawns taken after weaning as a result of the suckling period are presented in *(Table 2)*. While the average body weight of male fawns was 10.90 kg, the average body weight of female fawns was 9.71 kg. While the average chest circumference of male fawns is 53.10 cm, it is 52.03 cm for females. While the average shoulder height of female fawns was 49.70 cm, this height reached 51.53 cm for male fawns. There are no significant differences between genders and body measurements (P>0.05).

The differences between the body measurements at the end of the suckling period and the body measurements taken after birth during the 165-day suckling period are presented in *(Table 3)*. Male fawns gained an average live weight of 9.17 kg during this period, while female fawns gained an average live weight of 8.07 kg. This means an average daily live weight gain of 55.58 g in males during the suckling period. In female fawns, there was an average daily live weight gain of 48.91 g during the suckling period. While the abdominal circumference of male

Table 4. *Correlation coefficients between body measurements of males (above diagonal) and females (below diagonal) after weaning*

Measurements	BW (kg)	BL (cm)	CC (cm)	CD (cm)	AC (cm)	SH (cm)
BW (kg)		0.762**	0.945**	0.736**	0.755**	0.806**
BL (cm)	0.532*		0.672**	0.690**	0.593*	0.579*
CC (cm)	0.800**	0.451		0.790**	0.783**	0.813**
CD (cm)	0.587*	0.613*	0.464		0.713**	0.775**
AC (cm)	0.692**	0.604*	0.714**	0.451		0.684**
SH (cm)	0.728**	0.548*	0.495	0.895**	0.463	

*BW: Body Weight, BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth ** P<0.01*

Table 5. *Correlation coefficients between body measurements after weaning*

Measurements	BL (cm)	CC (cm)	CD (cm)	AC (cm)	SH (cm)
BW (kg)	0.707**	0.910**	0.715**	0.654**	0.785**
BL (cm)		0.615**	0.670**	0.588**	0.597**
CC (cm)			0.730**	0.707**	0.714**
CD (cm)				0.579**	0.793**
AC (cm)					0.565**

*BW: Body Weight, BL: Body Length, CC: Chest Circumference, CD: Chest Depth, AC: Abdominal Circumference, SH: Shoulder Heigth ** P<0.01*

Table 6. *Multiple regression equations for chest circumference, shoulder heigth and body length for predicting live weights of Gazella marica*

Dependent Variable	Gender	Model	Intercept	Independent Variables			$^{b}R^2$	^{a}RSD	P
				CC	SH	BL			
Body Weigth	Male	1	-16.40	0.5	-	-	0.89	0.95	<0.01
	Female	1	-8.7	0.35	-	-	0.64	0.80.	<0.01
		2	-13.83	0.26	0.20	-	0.79	0.87	<0.01
	General	1	-15.73	0.5	-	-	0.83	0.94	<0.01
		2	-20.27	0.39	0.2		0.87	0.93	<0.01
		3	-19.82	0.35	0.16	0.08	0.88	0.94	<0.01

CC: Chest Circumference, SH: Shoulder Heigth, BL: Body Length, ^{a} RSD: Residual standard deviation, ^{b} R^2: Determination coefficient. Model 1: Predictors: (Constant), Chest Circumference, Model 2: Predictors: (Constant), Chest Circumference, Shoulder Heigth, Model 3: Predictors: (Constant), Chest Circumference, Shoulder Heigth, Body Length

fawns increased by 24.87 cm on average, the abdominal circumference of female fawns increased by 23.07 cm. There are no significant differences between genders and body measurements (P>0.05) *(Table 3)*.

The correlation between body measurements in male and female fawns after weaning is presented in *(Table 4)*. In male fawns, there is a very high positive correlation (0.945) between body weight and chest circumference, while there is a medium positive correlation (0.579) between body length and shoulder height. There is a high positive correlation (0.783) between abdominal circumference and chest circumference (P<0.01). There is a high positive correlation (0.800) between body weight and chest circumference in female fawns. However, it is slightly lower than that of males. However, in female fawns, the highest correlation (0.895) is between chest depth and shoulder height. The lowest correlations are between chest circumference and body length (0.451) and between abdominal circumference and chest depth (0.451), with a near-medium correlation (P<0.01).

There is a very high positive correlation (0.910) between body weight and chest circumference. There is a moderate

positive correlation (0.565) between shoulder height and abdominal circumference. There is a moderate positive correlation between body length and chest circumference, chest depth, abdominal circumference, and shoulder height, respectively (0.615, 0.670, 0.588, 0.597) (P<0.01) *(Table 5)*.

In male gazelle fawns, body weight can only be estimated based on chest circumference. Body weight can be predicted by chest circumference at a rate of 89% (P<0.01). In female gazelle fawns, body weight can be estimated by both chest circumference, chest circumference and shoulder height. In female offspring, 64% of body weight can be predicted by chest circumference (P<0.001). Body weight can be predicted by 79% with shoulder height and chest circumference measurements (P<0.01). In general, 83% of body weight can be estimated by chest circumference measurements (P<0.01). In general, body weight can be estimated as 87% by measurements of shoulder height and chest circumference (P<0.01). Body weight can be predicted by 88% with body length, shoulder height and chest circumference (P<0.01) *(Table 6)*.

Discussion

In this study, no statistically significant difference was found between body measurements and gender in gazelle fawns after birth and after the suckling period. There were small differences between males and females, but there was no statistical difference. The body size of male fawns is larger than females. This is because male fawns are thought to grow faster and more dominant than females. After birth, the average body length of female fawns was longer than male fawns, while after weaning, the average body length of males was longer than females. It has been reported that the average weight of the newborn fawns is around 1.84 kg for females and 1.95 kg for males. It has also been reported that the average birth weight of single-born is 1.92 kg and the average birth weight of twins is 1.82 [13]. In addition, Martin et al.[14] reported male gazelle fawns of *Gazella arabia* are born heavier than females. The results of Gürler et al.[13] and Martin et al.[14] are similar to each other. However, our results showed that the average body weights of male and female fawns were lower than those of reported by Gürler et al.[13] and Martin et al.[14]. One of the reasons for this is that the study maden Gürler et al.[13] included fawns born between 1-3 days. This may be due to the fact that the study we studied was on fawns that were 1-2 days old. These results show that if gazelle fawns can be detected at the first day of age, they will have lower birth weights and body measurements. The body weight, chest circumference and shoulder height results we found after weaning are similar to the studied on young gazelles maden by Gürler et al. [13]. Gazelle fawns can reach the same body size as youngsters in 5.5 months. Since Gürler et al.[13] measured fawns and juveniles in the same period, the gazelles specified as young were those that had completed one year of age. Gazelles raised in semi-captive conditions will have higher body weight and body measurements starting from 5.5 months, thanks to good care and feeding conditions [13]. After 165 days of suckling peiod, the fawns gained an average daily live weight of 49-55 g. Post-weaning body changes in *Capreolus capreolus* fawns in roe deer species in two different regions were reported to be 0.017 kg/day in Chizé and 0.014 kg/day in Dourdan for the October-March period [22]. Daily live weight gain after weaning in Central Anatolian Merino (CAM) and Malya sheep lambs was reported as 310 g and 280 g, respectively [23]. In Kıvırcık lambs, an average daily live weight gain of 0.203 kg was achieved during the 75-day fattening period from birth [24]. In addition, if the feed given to gazelle fawns is made as a result of a controlled study, the feed conversion rate can be found.

Studies on some sheep breeds have reported that morphometric measurements differ from each other [25]. In a study done to by Topai and Macit [18] estimating body weight in Morkaraman sheep, it was reported that the highest correlation (0.867) was found between heart circumference and body weight, while the lowest correlation (-0.014) was between chest circumference and body length. In another study, it was reported that there was a high positive correlation (0.078) between body weight and body length in Tahalli sheep [26]. In a study done on dwarf goats in Ghana, it was reported that there was a medium correlation (0.44-0.59) between body weight and body length, shoulder height, chest/heart circumference, shoulder tip width, rump length and rump width [19].

In studies done on domestic sheep in Pakistan, it was reported that there was a high level of positive correlation between chest circumference and body weight (0.744) and a medium significant relationship between shoulder height and body weight (0.419) [27]. In their study, Esen and Elmaci [28] reported that the highest correlation between body measurements and live weight in meat type lambs was chest circumference. In addition, body weight estimation based on body measurements varies between 74% and 92% according to race. In a study done on the species *Gazella rufifrons kanuri*, it was reported that there was a positive correlation between body weight, body length and horn length [22]. It is reported that *Gazella dorcas* has a correlation coefficient of 0.79 between body length and chest circumference, and *Redunca redunca* antelope has a correlation coefficient of 0.99. The rate of estimating the body weight of *Gazella dorcas* based on its body measurements varies between 78% and 89% [20]. In a study maden on Florida White-tailed Deer *(Odocoileus virginianus),* it was reported that chest circumference was the single best determinant of body mass, and sub-gender,

age and sub-species information did not affect determining body mass [29]. When estimating body weight using chest circumference, shoulder height and body length; Body weight cannot be estimated based on chest depth and abdominal circumference. Since these values constantly change, it becomes difficult to obtain accurate results [30]. When examined in general, body weight estimation can be made in gazelle fawns with 3 different models. These are chest circumference, chest circumference and shoulder height respectively, and finally, chest circumference, shoulder height and body length and body weight are estimated. In the study done on Gazella dorcas, it was reported that the estimated body weight according to age and gender was 76% [30]. In Eastern Anatolian Red calves, live weight estimation can be estimated at a level of 95% based on chest circumference from birth to the 6th month [31]. In a study done on Madura cattle breeds, it was reported that the estimated growth curve with body weight was 68-70% [32].

As a result, selecting new individuals as breeders according to their phenotypic characteristics is very important for herd's future. The offspring, consisting of individuals with high body sizes, will carry the characteristics of their parents [33-35]. In addition, selecting better individuals under the same care and feeding conditions will result in higher genetic progress. This situation can also be used in gazelles. For example, in male gazelles, selecting individuals with larger body sizes and the same care and feeding conditions will facilitate selection. It will be beneficial in obtaining new individuals with high average scores within the herd. When female individuals reach sufficient body size, it will be easier for them to be used in breeding. A better selection can be made by genetic analysis in addition to body measurements [36]. In this way, the transmission of hereditary diseases found in breeding individuals to new individuals can be prevented. Selecting breeding individuals based on the high correlations and prediction values we have obtained will ensure faster genetic progress in the herd.

Declarations

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

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Research Article

Impact of Early Qualitative Feed Restriction and the Provision of Barrier Perch on Morphometric and Biomechanical Measurements of Bones in Broiler Chickens

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Abstract

An experiment investigated the effects of early qualitative feed restriction and barrier perch provision on the morphometric and biomechanical measurements of the bones in broiler chickens. A total of 504 one-day-old Ross 308 male broiler chickens were randomly assigned to a completely randomized design with a 2×2 factorial arrangement (three replicate pens/group;42 chickens/pen) of qualitative feed restriction (presence-QFR+/absence-QFR-) and the provision of barrier perch (presence-BP+/absence-BP-). On days 21 and 42, morphometric and biomechanical parameters were measured. As a result of this study, barrier perch presence showed no significant impact on morphometric and biomechanical measurements. In the QFR- group, the weight and length of femur bones in 42-day-old broiler chickens were found to be higher (P<0.0001, P=0.034, respectively), alongside increased weight (P=0.001), inner (mediolateral P=0.002 and craniocaudal P=0.040) and outer (mediolateral P=0.047) diameter of the tibiotarsus bones. Furthermore, weight-length index values of the femur and tibiotarsus bones were higher (P=0.001, P<0.0001, respectively) in the QFR- group, while robusticity index values were lower (P=0.029, P=0.006, respectively) on the 42nd day. Regarding biomechanical parameters, the ultimate force level of tibiotarsus bones was statistically higher (P=0.030) at 42 days in the QFR- group. In summary, early protein and energy restriction caused slight decreases in some measurements of the femur and tibiotarsus bones. Strong correlations were observed between specific morphometric and biomechanical parameters, demonstrating their potential to predict biomechanical measurements.

Keywords: Animal welfare, Barrier perch, Bone, Broiler chicken, Diluted diet, Three-point bending

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Introduction

Poultry meat is an economical, readily available [1], and globally consumed source of protein [2]. In the past five decades, chicken meat production has increased fivefold, parallel to the doubling of the global population. FAOSTAT forecasts indicate that this upward trajectory in poultry meat consumption will persist, driven by factors like affordability, consistent quality, adaptability, and high protein/low-fat content [3]. In order to meet this rising demand within the modern poultry industry, both the production amount has been increased, and hybrids have been developed that attain slaughter weight faster, and more breast muscle yield [4]. Rapid body growth and exacerbated development of the pectoralis major muscle have shifted broiler chickens' center of gravity and altered the broiler chickens' posture and load on the skeleton, leading to biomechanical imbalances [5-7]. This situation, resulted in increased load on the leg bones, particularly the femur and tibiotarsus [8]. This imbalance in muscle and skeletal development has led to issues like compromised leg health and lameness in modern broiler strains. These skeletal problems along with poor leg health, can lead to culling, mortality, reduced feed efficiency, and growth redaction, and are recognized as the leading cause

of economic losses in broiler chicken production [9]. Therefore, in recent years, studies have focused on nutritional and environmental factors to minimize the economic losses and animal welfare concerns caused by leg health problems in broiler chickens [6].

In modern broiler strains, a high growth rate leads to rapid periosteal bone deposition, compromised mineralization, altered biomechanical properties, and increased cortical bone porosity [10,11]. Low levels of mineralization have been linked to higher fracture risk, as the degree of bone mineralization directly impacts bone strength [12]. Modulating the growth rate through feed restriction has been demonstrated to enhance bone mineralization and skeletal development, thereby positively impacting bone quality [10]. Thus, various feed restriction techniques are utilized during the early stage of life to mitigate issues related to the excessive growth rates of modern strains [13-16]. These techniques include quantitative feed restriction methods, such as daily feed restriction, skip-a-day feeding, and time-restricted feeding, as well as qualitative feed restriction methods, such as diet dilution, low-protein diets, and low-energy diets [14]. Quantitative feed restriction involves limiting the amount of feed provided to animals daily [17]. Qualitative feed restriction is an effective approach that involves nutrient dilution in the diet [17-19]. Because this strategy mitigates the adverse impacts of either starvation or chronic starvation on broiler welfare [11].

Another negative result of a high growth rate is the increase in the time spent sitting in broilers together with the increase in growth rate and decrease in locomotor activities [20]. Therefore, there has been a focus on increasing mobility levels to improve animal welfare in recent years. It has been reported that increased mobility may both have positive impacts on leg health and reduce the risk of ammonia burns in broiler chickens by improving litter conditions [21]. To this end, a multitude of studies have been carried out to explore the impacts of environmental enrichments like perches [22,23], barriers [24,25], straw bales [26,27], or platforms [28,29]. Nevertheless, the literature review revealed a scarcity of studies that specifically delved into the impacts of environmental enrichments on bone measurements.

To evaluate bone quality, researchers use invasive techniques, including bone-breaking force, bone mineral density, bone mineral content, and bone ash content [30,31], and non-invasive techniques, including dual-energy X-ray absorptiometry and various imaging approaches [32]. In addition, calculations based on morphometric measurements such as cortical index, robusticity index, and weight-length index (Seedor index) are also used to measure bone mineralization [33].

The study has been designed considering the issues observed in the poultry industry' described above. Given that normal bone development in healthy broiler chickens reaches its peak within the initial three weeks of life [34], it was hypothesized that slowing the growth rate during this period could promote better bone development. To this purpose, qualitative feed restriction was applied during the first three weeks of life. Barrier perch was preferred to increase the mobility level of broiler chickens, and therefore, it was placed between the feeder and the drinker. In addition, its widespread usability and cost in the chicken industry were considered. In line with the information, this study aimed to investigate the impacts of implementing early-life qualitative feed restriction to limit weight gain and the use of a barrier perch to enhance mobility on morphometric and biomechanical measurements.

Material and Methods

Ethical Statement

All experimental procedures conducted in this study received approval from the Local Ethics Committee for Animal Experiments at Aydın Adnan Menderes University (approval no: 64583101/2023/57).

Experimental Design and Groups

An experiment was conducted based on a completely randomized design involving a 2x2 factorial arrangement of groups. A total of 504 one-day-old Ross 308 male broiler chickens (initial body weights: 46.73±0.17) were randomly allotted four groups and three replicates (3 replicates/group; 42 birds/pen). The experimental design is explained in detail in *Table 1*. The groups were categorized based on the application of early qualitative feed restriction (qualitative feed restriction-QFR+/without qualitative feed restriction-QFR-) and the provision of barrier perches (the presence of barrier perch-BP+/the absence of barrier perch-BP-).

All diets used in the experiment were formulated based on corn and soybean meal. The diets were prepared in three phases: starter, grower, and finisher, following the recommendations provided in the Ross 308 commercial

Table 1. *Experimental design*[1]

QFR	BP	Replicates	Birds Per Pen	Total Per Group
-	-	3	42	126
-	+	3	42	126
+	-	3	42	126
+	+	3	42	126
Total				504

[1]*QFR: qualitative feed restriction, BP: barrier perch.*

hybrid catalog [35]. In the QFR- group, the broiler chickens were fed a starter diet containing 3000 kcal/kg metabolizable energy (ME) and 23% crude protein (CP) from day 0 to 10. From day 11 to 24, they received a grower diet with 3100 kcal/kg ME and 21.5% CP. Finally, from day 25 to 42, they were provided with the finisher diet containing 3200 kcal/kg ME and 19.5% CP. For the QFR+ group, the feeding regimen was modified during the first 21 days. The quantity of soybean meal and vegetable oil in the diet was reduced, while the inclusion of wheat bran was increased to 15%. As a result, the CP content of the diet was reduced by 20% (18.6% and 17.2% CP), and the ME level was reduced by 10% (2700 and 2790 kcal/kg ME). From day 21 to 42, the broiler chickens in the QFR+ group were fed the same diet as the QFR- group. In the groups with BP+, a wooden perch barrier measuring 1.8 cm in width and 5 cm in height was installed between the feeder and the drinker from the 3rd day to the 14th day of the experiment. From the 14th day until the end of the experiment, a barrier perch measuring 1.8 cm in width and 15 cm in height was used in the same location.

Housing and Management

Broiler chickens were raised in floor pens (measuring 2 m in width, 2 m in depth, and 0.75 m in height) and located at the Poultry Research Unit, Aydın, Türkiye, for 42 days. Each pen was furnished with two drinker lines and two round feeders, ensuring ad libitum access to feed and water. Bedding material consisting of wood shavings (6 cm in depth) was utilized. The stocking density (33 kg/m^2) and lighting regimen (24 h of light for the initial seven days and 18 h of light with 6 h of darkness for the subsequent days) were established following the European Union Directive (2007/43/EC) [36]. The broiler chickens were maintained under optimal management conditions, which included temperature and humidity control [37].

Data Collection and Measurement Procedures

On day 21, a total of 60 broiler chickens (15 chickens per group), and on day 42, a total of 80 broiler chickens (20 chickens per group) were randomly selected and slaughtered. A total of 140 right femur and tibiotarsus bones were collected, and the surrounding tissues were removed. These bones were then stored in a deep freezer at -20°C for further measurements.

Morphometric measurements: Geometric properties (length, weight, mediolateral external diameter-DE_{ML}; mediolateral internal diameter-DI_{ML}; craniocaudal external diameter-DE_{CrCau}; craniocaudal internal diameter-DI_{CrCau}) and index measurements (cortical index-CI; weight-length index-WLI; robusticity index-RI) were determined on the tibiotarsus bones. The femur bones were measured for weight, length, DE_{ML}, RI, and WLI. The weights of the bones were measured using a digital weight scale (Scaltec SBP52, Heiligenstadt, Germany), while the length and diameter were measured using a digital caliper (Mitutoyo, Model No: CD-15CP, Code No: 500-181 U, Absolute Digital Caliper, Tokyo, Japan). Index measurements of the bones were calculated using the following formulas [38].

$$Cortical\ index\ (CI) = \frac{ML\ external\ diameter - ML\ internal\ diameter}{ML\ external\ diameter}\ x\ 100$$

$$Weight - length\ index\ (WLI) = \frac{bone\ weight\ (mg)}{bone\ length\ (mm)}$$

$$Robusticity\ index\ (RI) = \frac{bone\ length}{\sqrt[3]{bone\ weight}}$$

Biomechanical measurements: The right tibiotarsus bones (n=140) underwent a three-point bending test to evaluate their biomechanical properties. These tests were performed using a three-point bending test apparatus (ANSI/ASAE S459) on the Zwick/Roell Z 0.5 (Zwick Roell, Ulm, Baden-Württemberg, Germany) testing device at Aydın Adnan Menderes University Agricultural Research Center (TARBİYOMER). The span length was set at 50 mm. The device operated at the speed of 10 mm/min, with a preload force of 2 N applied. The testXpert II software was used to record the measured data. Data on bone deformation under pressure were obtained graphically, and the following parameters were determined from graph [39].

Ultimate force (F_{max}): The maximum force applied to the bone at the time of fracture. It's extrinsic strength properties of bone (Newton-N).

Stiffness (S): The resistance required to flex the bone is calculated using the slope of the elastic portion of the force-deformation curve. It's intrinsic properties of bone (N/mm).

Ultimate strength (σ): The maximum strength that a bone can withstand. It's intrinsic properties of bone (MegaPascal-MPa).

Elastic modulus (E): The resistance required to flex the bone is calculated using the slope of the elastic portion of the stress-strain curve. It's intrinsic properties of bone (MegaPascal-MPa).

Moment of inertia (I_x): Calculated based on the cross-section of the bone (inner and outer bone diameter at the bending point) to determine the bending strength and elastic modulus of the bone (mm^4).

Statistical Analysis

The data obtained from the study were analyzed using the SPSS software package (version 22.0, SPSS Inc., Chicago, IL, USA). To assess the normal distribution of the variables, the Shapiro-Wilk test/Kolmogorov-Smirnov test was employed. Non-normally distributed variables were subjected to logarithmic, square, and reverse transformation. The assumption of homogeneity

of variances was evaluated using Levene's test. Bone measurements were subjected to analyses using a general linear model procedure, and means were compared using the least square difference (LSD) method. The experimental model for the design was defined as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + E_{ijk}$$

Where Y_{ijk}=the observed value, μ=the overall mean, α_i=the effect of qualitative feed restriction (QFR- and QFR+), β_j= the effect of barrier perch (BP- and BP+), $(\alpha\beta)_{ij}$= the interaction between qualitative feed restriction and barrier perch, and E_{ijk}= the test error per observation. Since the interaction between groups was determined to be insignificant for the investigated traits, it was not included in the tables.

The correlation analyses and scatterplots were carried out between biomechanical measurements and geometric properties/index measurements of the tibiotarsus of broiler chickens aged 42 days old using the ggpairs function from the GGally package (version 2.1.2) of RStudio software (version 4.3.1, Inc, Boston, MA, USA). P value less than 0.05 was considered statistically significant.

Results

Morphometric Measurements

To determine the effect of feed restriction applied during the first 21 days, lower values were obtained in the group of QFR+ in all geometric properties made on the femur and tibiotarsus bones of the 21-day-old broiler chickens *(Tables 2, Table 3)*. The differences detected in all measurements except the length and internal diameter measurements (length, DI_{ML} and DI_{CrCau}) of the tibiotarsus were found to be statistically significant (femur: weight P<0.0001; length P=0.012; DE_{ML} P<0.0001; tibiotarsus: weight P<0.0001; DE_{ML} P<0.0001; DE_{CrCau} P<0.0001). Similarly, in the

Table 2. *Effects of QFR and BP on geometric properties of femur in broiler chickens*[1,2,3]

Day	Measurements[4]	Qualitative Feed Restriction			Barrier Perch			SEM
		QFR-	QFR+	P	BP-	BP+	P	
21st day	Weight (g)	4.92	4.09	<0.0001	4.57	4.44	0.450	0.12
	Length (mm)	50.31	48.70	0.012	49.70	49.39	0.658	0.46
	DE_{ML} (mm)	6.50	5.79	<0.0001	6.16	6.12	0.724	0.08
42nd day	Weight (g)	18.92	17.40	<0.0001	18.09	18.23	0.721	0.29
	Length (mm)	76.37	75.30	0.034	75.82	75.85	0.943	0.35
	DE_{ML} (mm)	10.51	10.21	0.059	10.33	10.39	0.737	0.11

[1] *Data presented as the least square means;* [2] *The sample size is 60 on the 21st day and 80 on the 42nd day;* [3] *The interaction between groups was not significant for investigated traits (P>0.05);* [4] DE_{ML}*: medio-lateral external diameter*

Table 3. *Effects of QFR and BP on geometric properties of tibiotarsus in broiler chickens*[1,2,3]

Day	Measurements[4]	Qualitative Feed Restriction			Barrier Perch			SEM
		QFR-	QFR+	P	BP-	BP+	P	
21st day	Weight (g)	7.00	6.00	<0.0001	6.48	6.51	0.914	0.17
	Length (mm)	69.88	68.45	0.130	69.51	68.83	0.468	0.66
	DE_{M_L} (mm)	6.03	5.36	<0.0001	5.72	5.68	0.735	0.08
	DI_{ML} (mm)	2.75	2.66	0.254	2.73	2.69	0.668	0.06
	DE_{CrCau} (mm)	5.23	4.76	<0.0001	5.05	4.94	0.337	0.08
	DI_{CrCau} (mm)	2.60	2.55	0.568	2.58	2.57	0.843	0.06
42nd day	Weight (g)	26.09	24.29	0.001	25.25	25.13	0.811	0.36
	Length (mm)	107.62	106.85	0.248	106.91	107.56	0.326	0.46
	DE_{ML} (mm)	10.10	9.75	0.047	9.90	9.95	0.753	0.12
	DI_{ML} (mm)	5.76	5.37	0.002	5.51	5.62	0.368	0.08
	DE_{CrCau} (mm)	8.39	8.12	0.064	8.31	8.20	0.456	0.10
	DI_{CrCau} (mm)	5.14	4.90	0.040	5.04	5.00	0.728	0.08

[1] *Data presented as the least square means;* [2] *The sample size is 60 on the 21st day and 80 on the 42nd day;* [3] *The interaction between groups was not significant for investigated traits (P>0.05);* [4] DE_{ML}*: medio-lateral external diameter,* DI_{ML}*: medio-lateral internal diameter,* DE_{CrCau}*: cranio-caudal external diameter,* DI_{CrCau}*: cranio-caudal internal diameter*

Table 4. *Effects of QFR and BP on index measurements of bones in broiler chickens[1,2,3]*

Day	Measurements[4]	Qualitative Feed Restriction			Barrier Perch			SEM
		QFR-	QFR+	P	BP-	BP+	P	
21st day	**Tibiotarsus**							
	CI	54.29	50.18	0.001	52.00	52.47	0.699	0.86
	WLI	99.69	87.34	<0.0001	92.82	94.21	0.602	1.87
	RI	3.66	3.78	<0.0001	3.74	3.70	0.118	0.02
	Femur							
	WLI	97.39	83.57	<0.0001	91.44	89.52	0.452	1.79
	RI	2.97	3.06	<0.0001	3.01	3.02	0.778	0.02
42n day	**Tibiotarsus**							
	CI	42.86	44.81	0.054	44.25	43.42	0.405	0.70
	WLI	242.28	227.18	<0.0001	236.06	233.40	0.521	2.91
	RI	3.63	3.69	0.006	3.65	3.68	0.199	0.02
	Femur							
	WLI	247.59	230.85	0.001	238.35	240.08	0.711	3.29
	RI	2.87	2.91	0.029	2.89	2.89	0.676	0.01

[1] Data presented as the least square means; [2] The sample size is 60 on the 21st day and 80 on the 42nd day; [3] The interaction between groups was not significant for investigated traits (P>0.05); [4] CI: cortical index, WLI: weight-length index, RI: robusticity index

geometric properties of the femur and tibiotarsus bones of the 42-day-old broiler chickens, higher values were observed in the group of QFR-. Statistically significant differences were detected in all geometric properties (femur: weight P<0.0001; length P=0.034; tibiotarsus: weight P=0.001; DE_{ML} P=0.047; DI_{ML} P=0.002; DI_{CrCau} P=0.040) except for the DE_{ML} of the femur and the length and DE_{CrCau} of the tibiotarsus.

In the tibiotarsus of 21-day-old broiler chickens, the CI was significantly higher (P=0.001) in the QFR- group, whereas in the tibiotarsus of 42-day-old broiler chickens, it was higher (P=0.054) in the QFR+ group *(Table 4)*. The WLI of tibiotarsus bones of 21 and 42-day-old broiler chickens with qualitative feed restriction was found to be lower (P<0.0001) while the RI was higher (P<0.0001; P=0.006, respectively). The WLI of the femur of 21 and 42-day-old broiler chickens with qualitative feed restriction was lower (P<0.0001; P=0.001, respectively) while the RI was higher (P<0.0001; P=0.029, respectively). The presence of the barrier perch did not affect the morphometric measurements (geometric and index) of the tibiotarsus and femur bones in 21 and 42-day-old broiler chickens.

Table 5. *Effects of QFR and BP on biomechanical measurements of tibiotarsus in broiler chickens[1,2,3]*

Day	Measurements[4]	Qualitative Feed Restriction			Barrier Perch			SEM
		QFR-	QFR+	P	BP-	BP+	P	
21st day	F_{max} (N)	166.46	123.43	<0.0001	147.53	142.37	0.512	5.53
	S (N/mm)	77.81	67.67	0.004	72.30	73.18	0.794	2.38
	σ (MPa)	81.96	84.14	0.450	82.28	83.83	0.589	2.02
	E (MPa)	1141.26	1503.32	<0.0001	1278.03	1366.55	0.346	65.87
	I_x (mm^4)	41.70	27.20	<0.0001	35.88	33.02	0.403	2.18
42nd day	F_{max} (N)	315.35	287.57	0.030	305.15	297.77	0.598	9.04
	S (N/mm)	87.67	84.12	0.208	84.45	87.34	0.303	1.97
	σ (MPa)	79.46	78.60	0.799	78.35	79.71	0.688	2.37
	E (MPa)	1644.29	1753.85	0.235	1621.94	1776.20	0.207	80.64
	I_x (mm^4)	261.23	231.88	0.076	250.06	243.05	0.669	11.54

[1] Data presented as the least square means;[2] The sample size is 60 on the 21st day and 80 on the 42nd day;[3] The interaction between groups was not significant for investigated traits (P>0.05); [4] F_{max}: ultimate force, S: stiffness, σ: ultimate strength, E: elastic modulus, I_x: moment of inertia

Biomechanical Measurements

In the tibiotarsus of 21-day-old broiler chickens, the ultimate force, stiffness, and moment of inertia were higher in the QFR- group (P<0.0001; P=0.004; P<0.0001, respectively), whereas ultimate strength and elastic modulus were higher in the QFR+ group (P=0.450; P<0.0001, respectively). When examining the biomechanical measurements of the tibiotarsus bones of 42-day-old broiler chickens, all parameters, except for ultimate force (P=0.030), showed statistically insignificant differences between the QFR groups. The presence of barrier perch did not impact the biomechanical measurements of the tibiotarsus in 21 and 42-day-old broiler chickens *(Table 5)*.

Correlation Analysis Between Morphometric and Biomechanical Measurements

The study adopted the correlation coefficient standards described by Hayran and Hayran [40], where coefficients ranging from 0.05 to 0.30 are considered insignificant, those between 0.30 and 0.40 are categorized as low, those between 0.40 and 0.60 are categorized as moderate, those between 0.60 and 0.70 are categorized as good, those between 0.70 and 0.75 are categorized as very good, and those between 0.75 and 1.00 are categorized as indicating a strong correlation. The correlation coefficients (r) between geometric properties and biomechanical measurements of 42-day-old tibiotarsus bones are presented in *Fig. 1*. A strong positive correlation was found between outer diameters (DE_{ML} and DE_{CrCau}) and moment of inertia (r=0.877, P<0.001; r=0.970, P<0.001, respectively). Conversely, elastic modulus and outer diameters had a strong negative correlation (r=-0.789, P<0.001; r=-0.863, P<0.001). These results suggest that as outer diameters increase, there is a corresponding increase in the moment of inertia while a decrease in elastic modulus is observed. It was found that DE_{ML} showed a good negative correlation with ultimate strength (r=-0.659, P<0.001), while DE_{CrCau} exhibited a moderate negative correlation with ultimate strength (r=-0.557, P<0.001). Conversely, there was a good positive correlation (r=0.643, P<0.001) between DE_{CrCau} and ultimate force. The analysis revealed a moderate negative correlation between DI_{ML} and ultimate strength (r=-0.501, P<0.001). Furthermore, there was a moderate positive correlation noted between DI_{ML} and moment of inertia (r=0.512, P<0.001), as well as between DI_{CrCau} and moment of inertia (r=0.522, P<0.001).

The correlation coefficients (r) between the index and biomechanical measurements of 42-day-old tibiotarsus bones are shown in *Fig. 2*. The correlations between CI and biomechanical measurements were observed to be

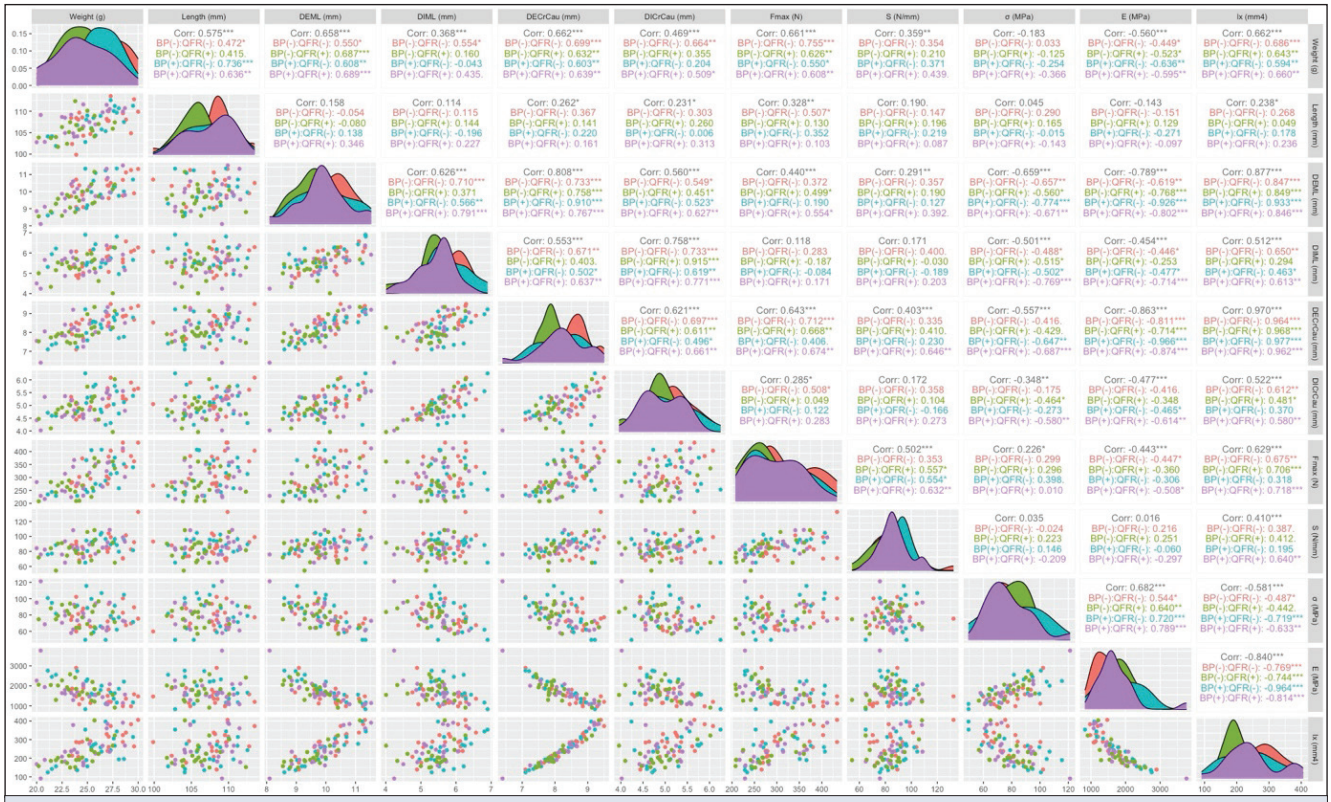

Fig 1. Scatterplots showing the correlation matrix results for biomechanical measurements and geometric properties of broiler chickens[1,2] (Created in RStudio version 4.3.1. using ggpairs function from the GGally package version 2.1.2). [1]DE_{ML}: medio-lateral external diameter, DI_{ML}: medio-lateral internal diameter, DE_{CrCau}: cranio-caudal external diameter, DI_{CrCau}: cranio-caudal internal diameter, F_{max}: ultimate force, S: stiffness, σ: ultimate strength, E: elastic modulus, I_x: moment of inertia; [2]A total of 80 tibiotarsus bones from 42-day-old broiler chickens were used in the correlation analysis

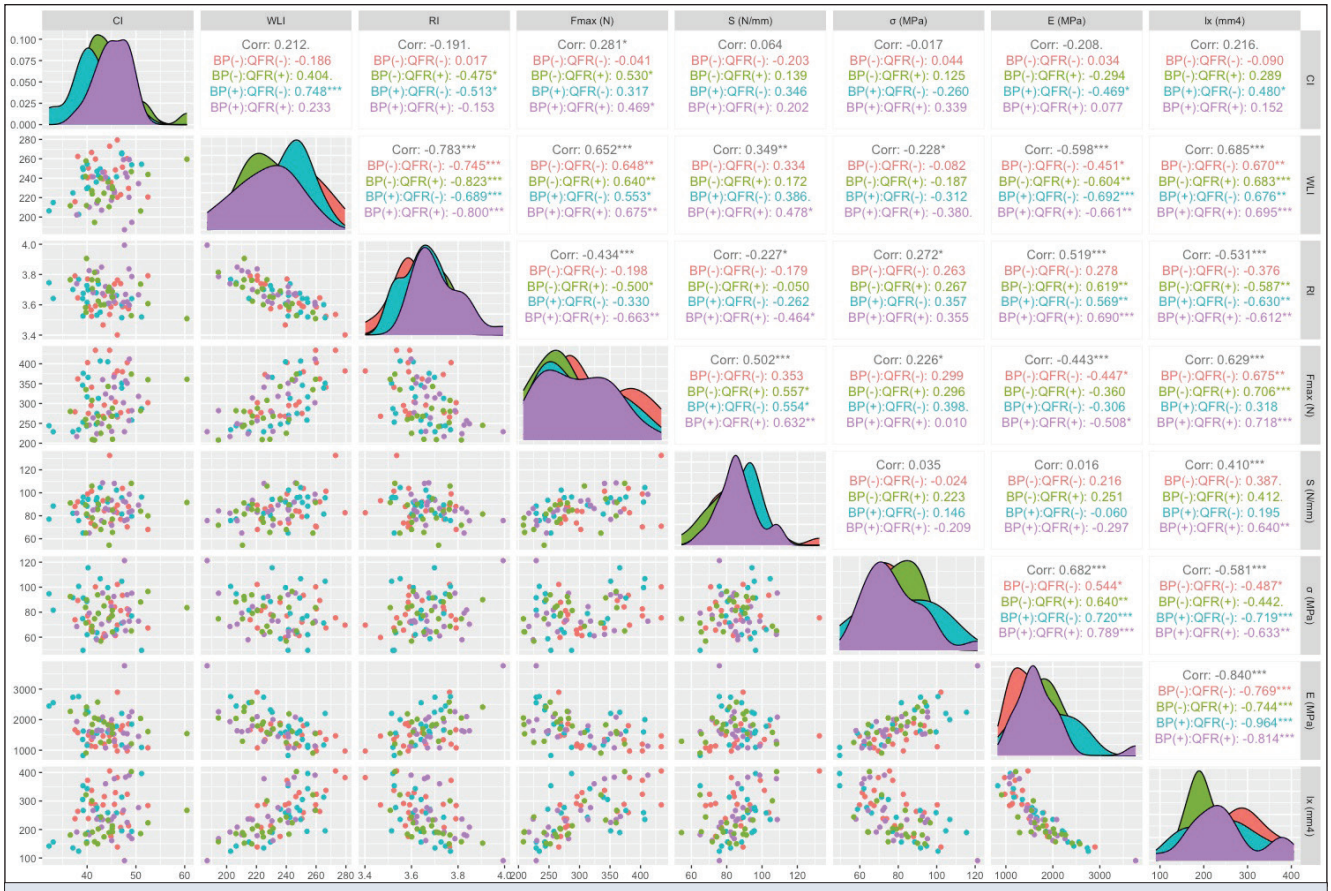

Fig 2. Scatterplots showing the correlation matrix results for biomechanical and index measurements of broiler chickens[1,2] (Created in RStudio version 4.3.1. using ggpairs function from the GGally package version 2.1.2). [1]CI: cortical index, WLI: weight-length index, RI: robusticity index, F_{max}: ultimate force, S: stiffness, σ: ultimate strength, E: elastic modulus, I_x: moment of inertia; [2]A total of 80 tibiotarsus bones from 42-day-old broiler chickens were used in the correlation analysis

low or insignificant (r<0.40). Conversely, a good positive correlation was identified between WLI and ultimate force (r=0.652, P<0.001), as well as moment of inertia (r=0.685, P<0.001). Additionally, a moderate negative correlation was found between elastic modulus and WLI (r=-0.598, P<0.001). RI and elastic modulus were observed to have a moderate positive correlation (r=0.519, P<0.001). A moderate negative correlation was identified between RI and ultimate force (r=-0.434, P<0.001), as well as moment of inertia (r=-0.531, P<0.001).

Discussion

In the research findings, qualitative feed restriction was observed to decrease the length and weight of femur bones of 42-day-old broiler chickens while leaving the diameter unaffected. Similar to the present findings, Yalçın et al.[41] reported that broiler chickens subjected to protein restriction exhibited shorter femur length. In contrast, Bruno et al.[42] reported that early qualitative feed restriction had no impact on the weight and length of femur bones but, resulted in a decrease in femur bone diameter in broiler chickens. Furthermore, Bruno et al.[43] indicated that early quantitative feed restriction did not affect the weight of femur bones, but led to reductions in both the length and diameter of femur bones in broiler chickens. In the study, it was found that qualitative feed restriction resulted in decreases in all geometric properties of the tibiotarsus bones of 42-day-old broiler chickens although the reductions in length and diameter (DE_{CrCau}) were not statistically significant. Consistent with the study findings, El-Faham et al.[44] and Pirzado et al.[45] reported that low energy levels did not affect the tibia length of 42-day-old broiler chickens. Similarly, Bruno et al.[42] found that early qualitative feed restriction did not influence tibia length. Conversely, Bruno et al.[43] stated that early quantitative feed restriction significantly reduced tibia length. Additionally, contrary to the present study findings, previous studies reported that tibia weight was not affected by low protein/energy levels [45,46] or early qualitative/quantitative feed restriction [42,43] in broiler chickens. Venalainen et al.[47] stated that tibia length, external diameter, and weight were greater in broiler chickens given high ME diets than in those given low ME diets. In the study, better results were obtained in 42-day-old broiler chickens in the group without feed restriction in terms of WLI and RI. Conversely, El-Faham et al.[44]

reported that energy restriction did not affect levels of WLI and RI. This discrepancy among study results might be related to differences in methods, duration, or levels of feed restriction. When the findings of the current trial were evaluated, it was observed that qualitative feed restriction reduced or tended to reduce all geometric properties of the femur and tibiotarsus on day 42. This situation is thought to be due to the limitation of growth caused by feed restriction. This notion is also evident from the 240.53 g body weight difference observed between the QFR groups on days 0-42 in the present study (data not shown).

The research findings align with previous studies that reported no significant effect on the presence of barrier perch on the weight, length, and diaphysis diameter of tibiotarsus bones of 42-day-old broiler chickens [22,48]. Similarly, Ventura et al.[49] observed in their study that the use of barriers did not affect the diameter and length of the tibia bones in broiler chickens. In contrast to these findings, Türkyılmaz et al.[50] stated that perch use significantly increased the broiler chickens' tibia weight, length, and diameter. Bizeray et al.[24] reported that the utilization of barriers did not have an impact on broiler chickens' tibia length but led to an increase in diameter. Consistent with the research results, Karaarslan and Nazlıgül [38] and Dereli Fidan et al.[48] noted that the provision of perch did not affect index measurements of the tibiotarsus bones (CI, WLI, RI) of 42-day-old broiler chickens. It is thought that the inconsistent findings might be due to differences in the designs and configurations of the perches used.

It was determined that implementing protein and energy level restrictions to decelerate the growth rate of broiler chickens did not result in the anticipated levels of difference in bone biomechanical measurements among the groups. The limited impact of the feed restriction is attributed to its application solely during the initial 21 days, followed by a return to a normal diet for the subsequent 21 days. The effect of early qualitative feed restriction on biomechanical measurements of tibiotarsus bones in 42-day-old broiler chickens was statistically insignificant, except for ultimate force. However, there was a visible trend towards an increase in the elastic modulus value. Unlike research outcomes, some researchers reported that protein and/or energy restriction impacts on breaking strength (ultimate force) were insignificant [45,46,51]. The inconsistency observed among study results may stem from variations in the methodologies, durations, or levels of feed restriction implemented. Further research exploring these factors in greater detail may help elucidate the underlying reasons for the discrepancies observed in the literature.

It was observed that the barrier perches, utilized to enhance bone strength by promoting mobility, did not achieve the anticipated effect on the biomechanical measurements of the tibiotarsus. Despite observing higher values of stiffness, ultimate strength, and elastic modulus in the tibiotarsus bones of broiler chickens raised in pens with barrier perches, these values fell below expectations. This outcome is attributed to the low levels of perching ratio of 2% observed in broiler chickens (data not shown). The effect of providing barrier perch on all biomechanical parameters of tibiotarsus bones in 21- and 42-day-old broiler chickens was statistically insignificant. It is consistent with the research results described by Bizeray et al.[24], who reported that the effect of barrier provision on the breaking strength and stiffness of tibia bones was statistically insignificant. Aksit et al.[22] revealed similar findings' showing that perch use had no significant effect on breaking strength. Similarly, Dereli Fidan et al.[48] observed that the presence of a perch did not affect the moment of inertia value. Moreover, Türkyılmaz et al.[50] stated that the use of perch did not affect the ultimate strength and elastic modulus. However, contrary to research findings, Türkyılmaz et al.[50] reported conflicting results stating that perch use led to a significant increase in ultimate force, stiffness, and moment of inertia. The inconsistencies in findings are thought to arise from variations in the designs and configurations of the perches employed in the studies.

The center of gravity in broiler chickens shifted towards the front of the body due to increased breast meat yield and growth rate, consequently altering the biomechanics of the leg bones [5]. For this reason, recent studies have primarily focused on the parameters of the tibia and femur bones. Yalçın et al.[41] reported a significant correlation between bone-breaking strength and bone weight, as well as bone length, suggesting that the bone-breaking strength can be predicted from these variables. Similarly, in line with the research findings, a positive and good correlation was found between bone weight and breaking strength (ultimate force). Additionally, in the present study, a substantial positive and good correlation was identified between weight and moment of inertia. Furthermore, according to the study results, as the outer diameter of the tibiotarsus bone (DE_{ML}, DE_{CrCau}) increased, a decrease in the level of elastic modulus and an increase in moment of inertia were observed. In light of these results, predictions can be made regarding ultimate force, moment of inertia, and elastic modulus based on the variables' weight and outer diameter.

The strength of a bone depends on its geometric properties, cortical thickness, porosity, and trabecular framework. Understanding bone strength often involves assessing bone density through techniques such as DEXA (Dual-energy X-ray absorptiometry) or using a variety of imaging methods. Additionally, geometric indices such as

the cortical index, robusticity index, and weight-length index also provide valuable information. Biomechanical tests, such as three-point bending, offer more precise insights into bone strength [23]. In this context, it was examined that the correlation between these indices and biomechanical measurements to gain a comprehensive understanding of bone strength [52]. Therefore, it should be evaluated in light of this information when examining the correlation levels between the RI and biomechanical measurements. Although there is a significant correlation between the RI and all biomechanical measurements, in terms of the degree of correlation, it was determined that there was only a moderate negative correlation with ultimate force and moment of inertia and a moderate positive correlation with elastic modulus. In line with this result, it can be said that as the RI value decreases, ultimate force and moment of inertia increase, and elastic modulus decreases. Similarly, significant correlations were identified between the WLI and all biomechanical measurements. Specifically, a significant positive correlation was observed with ultimate force and moment of inertia, alongside a moderate negative correlation with elastic modulus. It can be said that, as the WLI value increases, ultimate force and moment of inertia increase, but elastic modulus decreases. Conversely, no remarkable correlation was discerned between the CI and biomechanical measurements. In light of these results, predictions can be made regarding ultimate force, moment of inertia, and elastic modulus based on the variables of RI and WLI.

In conclusion, a strong/good correlation was found between some morphometric and biomechanical parameters (DE_{ML} and $I_x/E/\sigma$, DE_{CrCau} and $I_x/E/F_{max}$, weight and F_{max}/I_x, WLI and I_x/F_{max}). It is thought that these variables can be suggested to predict biomechanical measurements. It was determined that the presence of barrier perches did not affect bone morphometric and biomechanical measurements. It was found that protein and energy restriction applied during the early period resulted in a slight decrease in some morphometric and biomechanical measurements of the femur and tibiotarsus bones. Additionally, the inconsistent results reported in previous studies for all parameters examined in this trial suggest that bone shape in broilers may exhibit a high degree of individual variation. Further research exploring these factors may help elucidate the underlying reasons for the discrepancies observed in the literature.

Declarations

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

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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: SK was responsible for the conception and design of the study. SK, MK, and OT performed the experiments. SK, MK, and FSK were biomechanical analyses in this study. SK and AN performed statistical data interpretation. SK wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

Molecular Detection of Selective Virulence Factors of *Mycoplasma bovis* Local Isolates Involved in Bovine Mastitis

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Abstract

Mycoplasma bovis is believed to be a major cause of pneumonia, mastitis, and arthritis in cattle. Mastitis is the most prevalent production-related disease in dairy herds. The present study aimed to improve the current knowledge on the virulence factors of *M. bovis*-induced mastitis in dairy cows. To this end, sampling was done from farms in Chaharmahal and Bakhtiari province. The samples were monitored for growth in a specific PPLO medium in a CO2 incubator. Simultaneously, the DNA of the samples was extracted and PCR was used to detect M. bovis strains. After identifying susceptible isolates of *M. bovis*, P48, alpha-enolase, P81, and LppB genes were investigated in *M. bovis* positive PCR samples. In total, out of 204 samples, 11 isolates suspected of *M. bovis* were obtained (5.39%) via culture method and 21 positive samples (10.29%) were obtained through PCR. 57.14 (12/21), 66.66 (14/21), and 52.38 (11/21)% of the samples were positive concerning the presence of P48, alpha-enolase and LppB genes, respectively. P81 was not detected in any of the positive samples. The results of the present study showed that the presence of *M. bovis* in dairy herds in Chaharmahal and Bakhtiari could be considered among the factors that cause mastitis in cattle. alpha-enolase gene was found to be more abundant than the other selected genes, which could play an important role in the future strategic measure to develop preventive measures against mycoplasmal mastitis.

Keywords: Mastitis, *Mycoplasma bovis*, Virulence gene, Prevalence

Introduction

Mastitis can inflict significant harm upon farm animals, primarily manifesting as diminished milk production in lactating cows during their lactation period and leading to the extermination of affected animals [1,2]. In cases of acute mastitis, substantial losses, and costs are tied to the removal and mortality of farm animals. Meanwhile, subclinical forms of mastitis, often stemming from delayed disease diagnosis, result in pronounced reductions in milk production and quality during lactation. The subclinical variant is the most prevalent type of mastitis, which poses the most significant detriment to the livestock industry. The acute form of mastitis, though less frequent, holds significance, too, with its extent of impact varying across different countries and farming setups [3,4]. Notably, acute and subclinical mastitis can also detrimentally affect the fertility of farm animals. Mastitis syndrome is inherent in all mammals, yet its prevalence is heightened in cows due to milking-induced stress. The foremost pathological changes arising from mastitis, affecting both mammary tissue and milk, encompass alterations in milk color, presence of clots, elevation in somatic cell and white blood cell counts within milk, shifts in mammary gland tissue, and the occurrence of swelling, heat, pain, and detectable stiffness upon palpation of the mammary glands. It is essential to acknowledge that many instances of subclinical mastitis are challenging to identify readily [5,6].

The etiology of mastitis can be attributed to micro-organisms and their toxins, parasites, physical trauma, or chemical irritants. Bacterial infections, notably caused by microorganisms like *Streptococcus agalactiae*,

Staphylococcus aureus, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Escherichia coli*, constitute about 95% of mastitis cases. The remaining 5% stem from other microorganisms [1,4,7].

Mycoplasma, which belong to the Mollicutes class of bacteria and lack a cell wall, are ubiquitous and can potentially infect eukaryotes. *Mycoplasma bovis* is a destructive pathogen affecting beef and dairy cows globally, significantly contributing to mastitis, pneumonia, and osteoarthritis. These maladies collectively engender substantial economic losses within the dairy farming sector. *M. bovis* infections culminate in calf mortality, weight loss in surviving calves, and diminished milk production in dairy cows [5,8]. Of particular concern is mycoplasmal mastitis, particularly the infection of teats with *M. bovis*, which results in a surge in somatic cell count (SCC) in milk (from 10^7 to 10^9 cells per mL). This type of mastitis notably curtails milk production [6,9]. Importantly, mastitis arising from *M. bovis* infection endures over an extended period and predominantly presents as a subclinical mammary gland infection [2,4]. It has been documented that *M. bovis*-induced mastitis triggers a sustained increase in SCC and pro-inflammatory cytokines in milk; however, this persistent inflammatory response is not potent enough to eliminate *M. bovis* from the mammary gland [1].

Mycoplasma infections encompass conditions such as calf pneumonia and osteoarthritis, and additionally, they can give rise to genital disorders. Managing *M. bovis* infections effectively necessitates early intervention. Presently, immunoprophylaxis and antibiotic therapy have proven ineffective against *M. bovis* [2,10]. Exploring the surface protein alpha-enolase from *M. bovis* and its binding to plasminogen becomes pertinent [5,11]. Intriguingly, antibodies against alpha-enolase from *Streptococcus iniae* exhibit protective effects in models of zebrafish and mice, hinting at α-enolase's potential as a target for *M. bovis* vaccine production. The research underscores the correlation between the presence of the bacterial surface protein α-enolase and bacterial adhesion to host cells [1,12]. Owing to the marked antigenic diversity in *M. bovis*, proteins universally conserved across strains are likely the most promising vaccine targets. One illustrative example is the surface lipoprotein P48. This highly hydrophilic protein bears conserved motifs linked to complement activation and cytokine induction, rendering it an apt starting point for probing pathogenicity mechanisms and immune shifts during infection [1,13]. Notably, ten highly protected *M. bovis* proteins, namely PdhA, PepA, Tuf, P48, P81, OppA, LppA, PepQ, O256, and DeoB, have been recommended as suitable candidates for recombinant vaccine production. Surface antigens like P48, P81, and LppB Lipoproteins hold significance in this context [1,14].

In the the present study, the lack of comprehensive investigations into *M. bovis* virulence factors implicated in mastitis, coupled with the underexplored roles of P48, alpha-enolase, P81, and LppB in mastitis cases, underscores the need for further research. This study delves into the genes associated with each factor, focusing on the milk samples collected from mastitis cases.

Materials and Methods

Ethical Approval

The procedures were applied in the method section were in accordance with the experimental local ethics committee (Code of ethics: IR.SKU.REC.1399.005).

Sampling

Two hundred and four samples were gathered from eight semi-industrial cattle farms in the Chaharmahal and Bakhtiari province, specifically in Shahrekord city. For sampling, the cow's udder was washed and each cartier was wiped separately with a disposable napkin. The first three to four milkings were then discarded, and 50 mL of milk from each cartier was poured separately into a sterile falcon tube. After labeling, the samples were transferred to the laboratory on ice within a few hours. Clinical mastitis samples were procured from cows exhibiting at least one of the subsequent characteristics: (i) Visible abnormalities in the milk, such as discoloration, clots, or pus particles; (ii) Irregular physical conditions of the udder, including acute and diffuse swelling, warmth, pain, and redness; and (iii) General bodily responses encompassing varying degrees of anorexia, toxemia, dehydration, fever, increased heart rate, cessation of rumen movements, and listlessness. Before initiating the antibiotic treatment protocol, milk samples were obtained from the affected quarters.

Subclinical mastitis samples were acquired from quarters that tested positive using the California Mastitis Test (CMT). The CMT procedure was conducted as follows: following the exclusion of the initial three milkings from each quarter, starting from the fourth milking, approximately 2-3 mL of milk was deposited into the corresponding compartment of the CMT container (Maki Kala, product ID: MAKI-D-1041). Subsequently, 2-3 mL of CMT solution (purple bromocresol-containing detergent; KerbaTEST solution, KERBL Company) was introduced into each compartment. The CMT container was agitated for 10 sec to amalgamate the milk with the CMT solution. Ultimately, the outcomes of each Cartier test were independently interpreted and recorded.

Culture in PPLO Medium

The milk samples were centrifuged at 4000 rpm for 40 min. Then the sediment was first cultured in 5 mL of PPLO Broth medium for 24 h at 37°C and in the presence

of 10% carbon dioxide. After initial enrichment, 2 mL of media containing samples were cultured in the new PPLO Broth medium including basic medium plus 20% horse serum, 0.05% thallium acetate, and penicillin G (500 U/mL) and 5 0.0% of yeast extract and incubated in a CO_2 incubator for 5-7 days at 37°C, 90% humidity and 10% CO_2. The mediums were passaged again in PPLO Broth containing supplements two more times. At the end of each stage, 200 µL of the culture were transferred to the PPLO agar medium containing 20% horse serum, 0.05% thallium acetate, and 500 IU/mL penicillin G, and then incubated for 10 days at 37 degrees, 90% humidity, and 10% CO_2. After 72 h, for 10 days, the culture mediums were examined with an inverted microscope and with 10 X magnification in terms of growth and formation of "fried egg" shaped colonies.

DNA Extraction

After the centrifuging of the milk samples at 4000 rpm for 40 min, DNA extraction was done by DNP Kit (Cinacolon, Cat. No: EX6071) according to the manufacturer's instructions. Briefly, at first, 100 µL of protease buffer was added to 500 µL of milk sediment. After adding 5 µL of proteinase K and pipetting, the mixture was incubated for 30 minutes at 55°C. The lysis buffer was placed at 37°C for 10 minutes, then 400 µL of it was added to 500 µL of milk precipitate. In the next step, 300 µL of sedimentation buffer was added to the collection, vortexed for 5 sec, and then centrifuged at 12000 rpm for 10 min. The supernatant was discarded, then 1 mL of washing buffer was added and vortexed for 3 to 5 sec, and afterward, centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was placed at 65°C for 5 min to dry completely. The precipitate was dissolved in 50 µL of the solvent buffer by gently shaking and placing it at 65°C for 5 min. After centrifugation at 12000 rpm for 30 sec, the supernatant containing DNA was collected. The extracted DNA was kept at -20°C until the PCR test was performed.

PCR

The primer design was done using Gene Runner software for the sequences related to the genes encoding the selected virulence factors *(Table 1)*. For the molecular identification of *M. bovis*, species-specific primers [15] were applied. Briefly, PCR was performed at a total volume of 25 µL. Final concentrations in the reaction mixes were 12.5 µL of Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Cat. No.: A180301), 1.5 mM MgCl2, 20 pmol of each primer, 0.5 µg of genomic DNA, and 9.5 µL of deionized Sterile water. Positive (prepared from the Mycoplasma Research Center - Razi Vaccine and Serum Institute) and negative controls (distilled water) were included in each PCR reaction. The temperature conditions were set according to the data described in *Table 2*. After the completion of the PCR reaction, the PCR products were subjected to electrophoresis on a 1.5% agarose gel next to a 100 bp DNA Ladder (Genaxxon bioscience, Cat No: M3340). Then the positive samples were sent to Bioneer Co, Korea for sequencing.

Statistical Analysis

SPSS software version 21 (IBM corporation, USA) was applied for the investigation of the relationship between the variables using the Chi-square test.

Results

Statistical Results

After using SPSS software version 21 (IBM corporation, USA) for the investigation of relationships between the variables, applying the Chi-square test, no statistically significant relationship was found between the frequencies of virulence genes ($P>0.05$). There was a statistically significant difference between the prevalence of *M. bovis* among clinical and subclinical cases ($P<0.05$) using PCR test; but in case of culture method, this relationship was not significant ($P>0.05$). Furthermore no statistically significant relationships were found between the frequencies of virulence genes among clinical and subclinical cases ($P>0.05$). If *M. bovis* was detected by PCR in the first step, the investigation of virulence genes, was done. The relationship between the frequencies of virulence genes in cases isolated using culture method was not significant ($P>0.05$). Finally, a statistically significant relationship was found between the culture method and PCR in the detection of *M. bovis* ($P<0.05$).

Identification of Suspected Isolates of *M. bovis* Using Bacterial Culture

After culturing in PPLO agar and observing the morphology of the colonies, a number of isolates were considered as suspected *M. bovis*. After observation with a light microscope, the colonies suspected to be *M. bovis* had the characteristic morphology of this bacterium, i.e. "fried egg". Colonies were denser in the center and less dense in the periphery and were different in diameter *(Fig. 1)*. In general, the diameter of hemispherical egg colonies in PPLO agar medium varies from 10 to 500 µm. After the bacterial culture, 11 suspected isolates of *M. bovis* were isolated. Using bacterial culture, the frequency of *M. bovis* in cases of clinical and subclinical mastitis were one and 10 cases, respectively *(Table 3)*.

Molecular Identification of *M. bovis* by PCR

After the electrophoresis of the PCR products using a pair of specific primers to investigate *M. bovis*, the observation of a 1626 bp band in the tested samples and the positive control indicated the presence of *M. bovis* nucleic acid *(Fig. 2)*.

From the total number of 204 examined milk samples that were simultaneously tested by bacterial culture and PCR, 21 positive samples were detected for the presence of *M. bovis* nucleic acid (10.29%), using PCR. Among 21 PCR positive samples, only eleven samples were positive using bacterial culture. Furthermore, among the *M. bovis* positive PCR products, the frequency of infection in the clinical mastitis cases was 2 and in the subclinical mastitis cases was 19 *(Table 4)*.

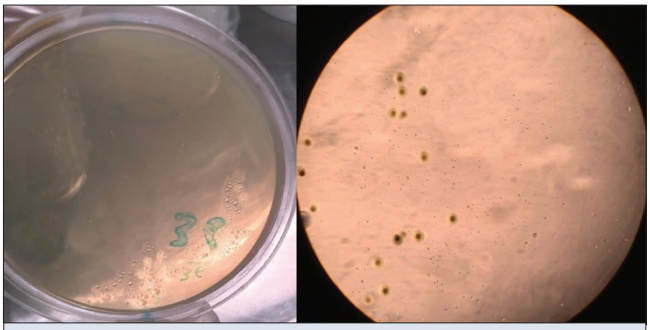

Fig 1. Suspected colonies of *M. bovis* observed on PPLO agar medium (magnification: 10X)

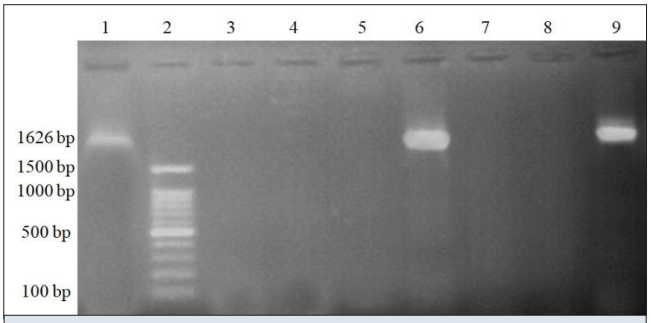

Fig 2. Electrophoresis gel of PCR product for detection of *M. bovis.* 1 and 6: Positive PCR products for M. bovis (1626 bp), 2: Ladder (100 bp), 7: Negative control, 3-5: negative PCR products, 9: Positive control (1626 bp)

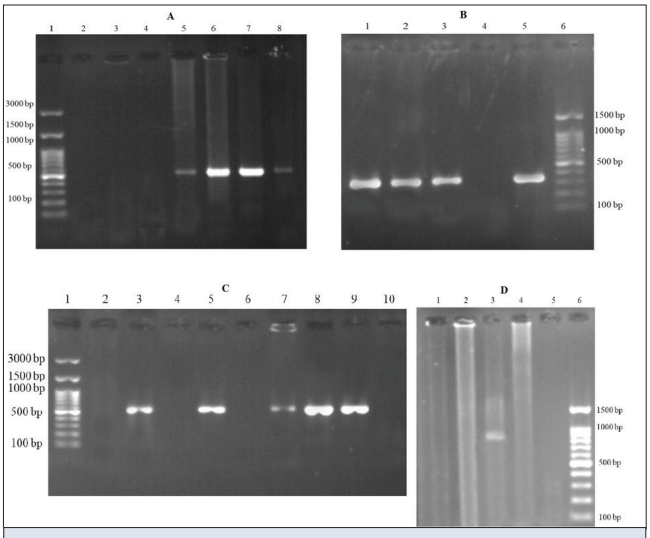

Fig 3. Electrophoresis of PCR product to detect P48, alpha-enolase, LppB and P81 genes. **A:** Detection of P48; 1: Ladder (100bp), 2: Negative control, 3, 4: Negative PCR products, 5-8: Positive PCR products for P48 gene (501 bp); **B:** Detection of alpha-enolase; 5 and 1-3: Positive PCR products for alpha-anolase (354bp), 4: Negative control, 6: Ladder (100bp); **C:** Detection of LppB ; 1: Ladder (100bp), 3,5,7-9: Positive PCR products for LppB gene (561bp), 6,2,4: Negative PCR product, 10: Negative control; **D:** Detection of P81; 1,2,4,5: Negative PCR products for P81 gene, 3: Positive PCR product for P81 (877bp), 6: 1: Ladder (100bp)

Investigation of Virulence Genes of *M. bovis* by PCR

In order to identify the selected virulence genes of *M. bovis*, PCR was performed on the DNA samples related to the cases that were found to be positive in the PCR test for the detection of *M. bovis*. After the electrophoresis of the PCR products for the detection of selected virulence genes, the bands with the sizes of 501 bp for P48, 354 bp for alpha-enolase, 877 bp for P81, and 561 bp for LppB were observed, indicating positive results for the presence of these genes. Unfortunately, in the case of the P81 gene, despite the application of several changes in the PCR protocol, only one suspected positive sample was obtained and the sample found was not confirmed by sequencing *(Fig. 3)*.

In total, out of 21 samples with positive results using the PCR test to identify *M. bovis*, after carrying out reactions related to the detection of virulence genes, 14, 11, and 12 samples were positive for the presence of the alpha-enolase, LppB, and P48, respectively *(Table 5)*.

Sequencing

In order to verify the products obtained in PCR reactions, sequencing was performed. 50 µL of the amplified fragments were sent to Gene Fanavaran Company for sequencing. The degree of homology (identity) of the sequence extracted from reading the PCR products with the sequences recorded in the gene bank and the expected amplicon was a good indication of the adequacy of PCR *(Fig. 4, Fig. 5, Fig. 6)*.

Table 1. *Sequence of primers used in this study*

Primer Name	Primer Sequence	Length	Tm (˚C)
MboF	TTACGCAAGAGAATGCTTCA	20	54
MboR	TAGGAAAGCACCCTATTGAT	20	54
P48F	TCAGTGCCATTGGTTGCTGCT	21	61
P48R	TGCTGCAGGTTCGCCAGTGC	20	65
AEF	ACCGGTGTTGCAAATGTGCCT	21	61
AER	TGGCAGTGGCAATGTGTGAGC	21	63
P81F	GGTAAGGGCGACGAGGACGA	20	65
P81R	CGGTTCCAACTTCTTCAGCGCCT	23	66
LppF	TGCTGCTAAATGTGGGGACGGT	22	64
LppR	CCTTTGCCAGGTTGTGCTGCT	21	63

Table 2. The thermal conditions for the amplification of the uvrC and virulence genes	
Gene	**PCR Cycling**
uvrC	94°C for 5 min followed by 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 60 s, followed by a final extension at 72°C for 10 min
P48	94°C for 5 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 35 s, followed by a final extension at 72°C for 10 min
alpha-enolase	94°C for 5 min followed by 30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min
P81	94°C for 5 min followed by 30 cycles at 94°C for 45s, 67°C for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 5 min
LppB	94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min

Table 3. Frequency of M. bovis in cases of mastitis using bacterial culture			
Mastitis Form	**Total Frequency**	**Frequency of *M. bovis* Positive Cases**	**Percentage of *M. bovis* Frequency**
Clinical	61	1	1.63
Subclinical	143	10	6.99
Total	204	11	5.39

Table 4. Frequency of M. bovis in cases of mastitis using PCR			
Mastitis Form	**Total Frequency**	**Frequency of *M. bovis* Positive Cases**	**Percentage of *M. bovis* Frequency**
Clinical	61	2	3.27
Subclinical	143	19	13.28
Total	204	21	10.29

Table 5. Frequency of M. bovis virulence genes using PCR				
Virulence Genes	**Frequency Among Clinical Cases**	**Frequency Among Subclinical Cases**	**Total Frequency of Positive Cases**	**Percentage of Positive Results**
Alpha-enolase	2	12	14	66.66
LppB	2	9	11	52.38
P48	2	10	12	57.14

Discussion

The economic losses inflicted by mastitis are not solely attributed to afflicted cows but also encompass diminished milk production [16]. Additional expenses encompass medication costs, costs tied to the period of milk abstinence, veterinary charges, supplementary labor expenses, compromised milk quality, supplementary diagnostic supply expenses, and a decrease in the quality and quantity of a cow's life. Hence, addressing this disease necessitates an approach encompassing epidemiology, prevention, and treatment [17]. In the current study, 204 milk samples were procured from semi-industrial farms in Shahrekord, cultured on PPLO Agar medium, isolating 11 suspected *M. bovis* isolates. Subsequently, a PCR test revealed the presence of *M. bovis* nucleic acid in 21 samples (10.29%). The PCR test proved more sensitive to *M. bovis* than the bacterial culture method [18]. This highlights the significance of incorporating molecular tests, such as PCR, in conjunction with phenotypic assessments like bacteriological culture to diagnose the presence of *Mycoplasma* in mastitis precisely.

This outcome is congruent with prior research, which asserts that 30% of mastitis milk may not yield bacterial growth in culture media. However, PCR remains adept at identifying bacteria even when growth is hindered or terminated [18].

Though the culture method is meticulous due to the gradual growth of the *Mycoplasma* genus, distinguishing between various species based on colony examination is intricate [19]. While bacterial culture remains a definitive and precise technique, its application in detecting *Mycoplasma* in milk can be impeded by inherent or induced inhibitors, necessitating many additives contingent upon the *Mycoplasma* species [14]. The PCR method's heightened sensitivity and specificity in discerning *Mycoplasma* species has been proven. PCR's sensitivity and specificity for detection of mastitis milk samples has been reported at 96.2% and 99.1%, respectively [12]. Furthermore, PCR sensitivity in detecting *M. bovis* in milk has been documented in a number of previous studies [20,21].

Internationally, various studies have utilized the uvrC gene as a diagnostic target for *M. bovis*. Thomas et al.[21] identified the uvrC gene as an amplification target for *M. bovis* strains. Later, Rosetti et al.[22] devised a novel Real-time PCR method for uvrC gene detection, facilitating the direct identification of *M. bovis* from milk and tissue samples sans DNA extraction. Similarly, Imandar et al.[15] identified *M. bovis* in cows with clinical mastitis using culture and PCR based on the uvrC gene. Those findings underscore the prevalence of *M. bovis* in Iranian dairy herds, potentially emerging as a principal cause of clinical mastitis in the country.

In congruence with the abovementioned research, the present study employed the uvrC gene to detect *M. bovis* species. The study's outcomes validate the PCR method's high sensitivity in detecting *M. bovis*, echoing the findings of earlier investigations. Notably, the prevalence of *M. bovis* varies across different regions in Iran. Moshkelani et al.[23] conducted a study utilizing a PCR test based on 16SrRNA gene fragment amplification to assess the

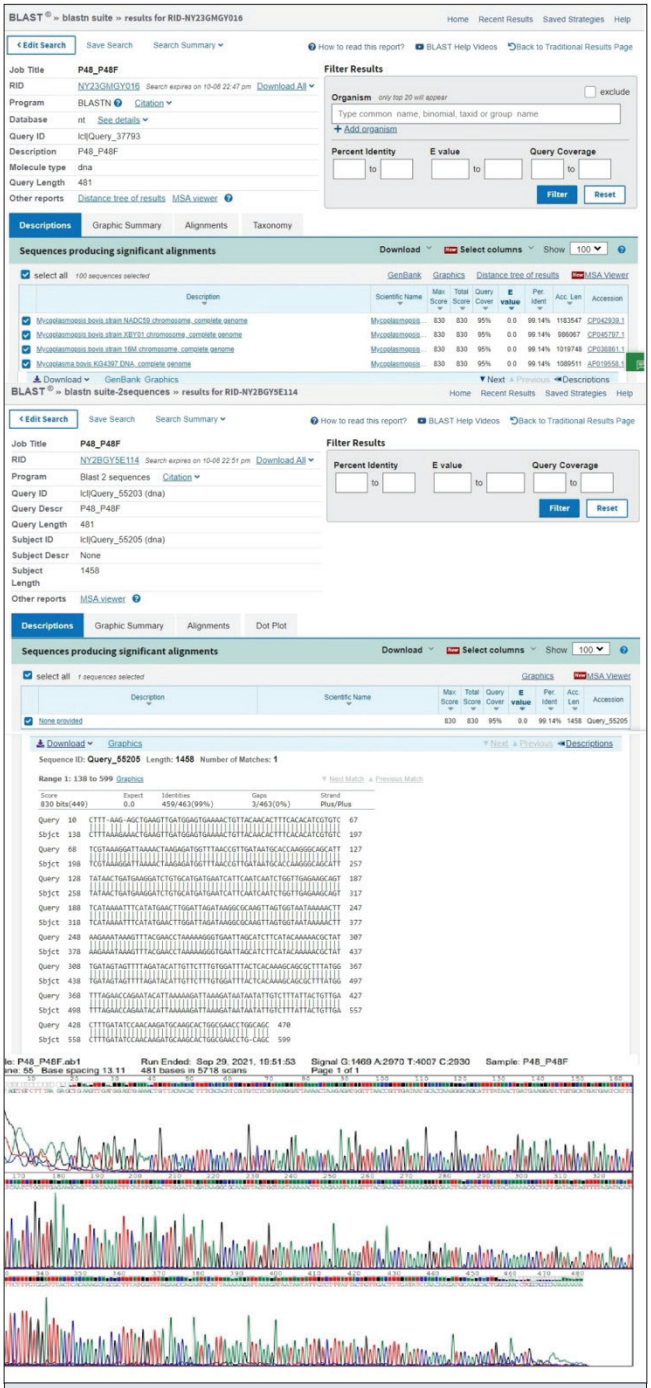

Fig 4. Alignment of the sequence extracted from the reading of the P48 gene PCR product with the sequences recorded in the gene bank and the expected amplicon

prevalence of subclinical mastitis in dairy herds in the Chaharmahal and Bakhtiari provinces. The prevalence of mycoplasmal mastitis was 48.67%.

A study was conducted in Shahrekord by Sharifzadeh et al.[24] using both culture and PCR methods to assess the abundance of *Mycoplasma* in industrial and semi-industrial dairy milk tanks. They found that the contamination of these tanks with *M. bovis* was 3.3%. The difference in the prevalence of mastitis due to *M. bovis* in the current study (10.29%) compared to previous studies in Chaharmahal and Bakhtiari may be attributed to various factors, including the sampling location, farming system type, hygiene levels on each farm, and sampling conditions. Moreover, differences in the specific target gene amplified for diagnosing *M. bovis* could also contribute to the variations observed.

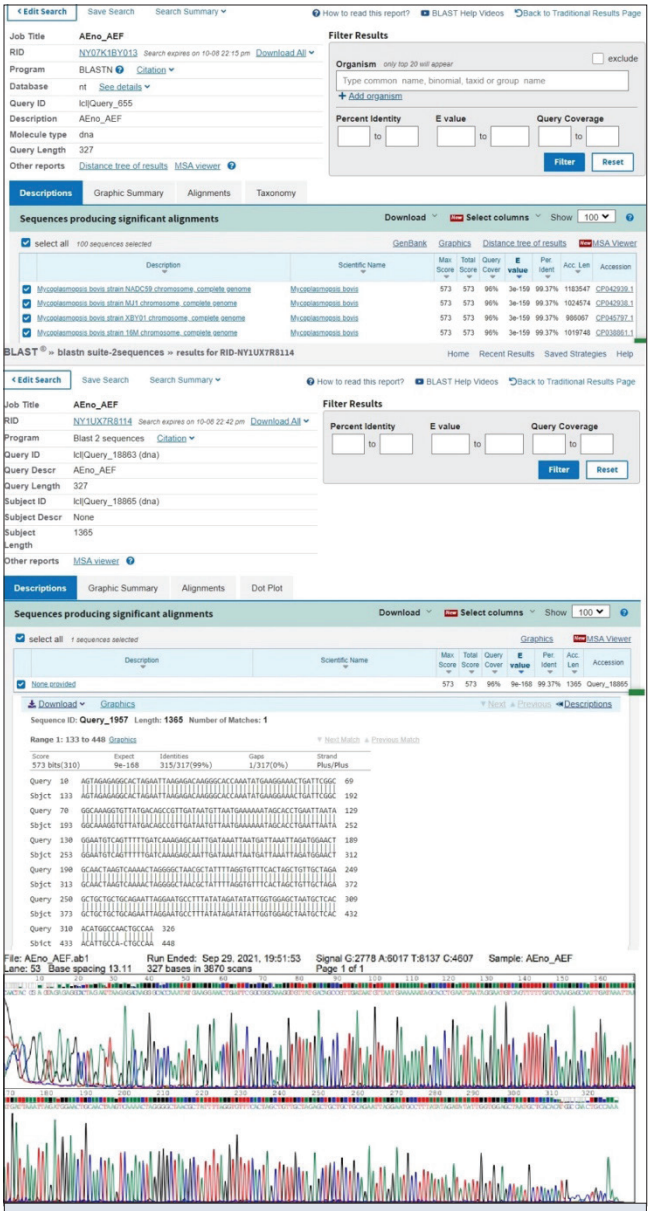

Fig 5. Alignment of the sequence extracted from reading the PCR product of the alpha-enolase gene with the sequences recorded in the gene bank and the expected amplicon

Given that hygienic practices during milking are more rigorously followed in industrial farms compared to semi-industrial ones in this province and that pathogen dilution is higher in milk tank samples, it was anticipated that the prevalence of *M. bovis* in the study conducted by Sharifzadeh et al.[24] would be lower than in the current study. At the time of sampling, we realized that,

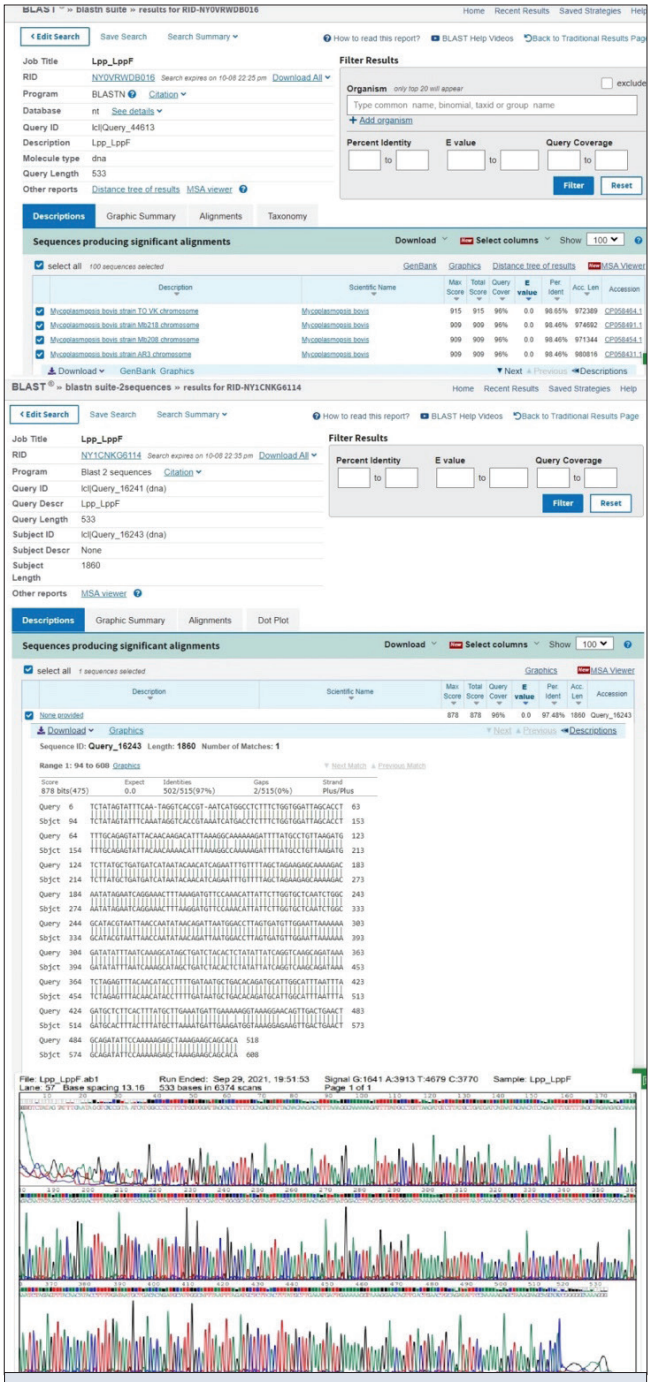

Fig 6. Alignment of the sequence extracted from the reading of the PCR product of the LppB gene with the sequences recorded in the gene bank and the expected amplicon

unfortunately, none of the necessary milking hygiene standards are observed in the sampling farms. The higher prevalence rate reported by Moshkelani et al.[23], compared to the current study, could be attributed to various factors. Notably, their study identified mycoplasmal mastitis in 50 dairy cows in general without limiting it to a specific species. Other factors that may have affected the prevalence include differences in management practices, choosing an adequate sample size, sampling times, and conditions.

Another study conducted in Ardabil examined 80 milk samples from dairy cows using culture in the Hyflick medium and immunoperoxidase testing. They found 39 samples (48.75%) of clinical mastitis cases were positive for *M. bovis* [25]. The methods employed for detecting *M. bovis* in study may have been effective in reporting a relatively high prevalence of *M. bovis*. Similarly, a study by Taleb Khan Grossi et al.[26] revealed an infection rate of 15.38% of mycoplasmal mastitis in dairy herds around Mashhad which is lower than prevalence rate reported by Moshkelani et al.[23], presumably due to a larger sample size and less incidence of antibiotic resistance in that year. Additionally, variation in dairy cattle breeding systems, milking hygiene within the herd and the timing and location of research can all lead to different results being obtained.

In the present study, the frequency of the alpha-enolase gene in the samples was higher than that of other selected genes, at 66.66%. Song et al.[27] introduced alpha-enolase as a virulence factor associated with *M. bovis* adhesion. Zhao et al.[28] further stated that specific highly conserved bacterial proteins, such as alpha-enolase, which are involved in metabolic regulation or cellular stress responses, also act as adhesives. *M. bovis*-encoded enolase is considered a pathogen-related factor that plays a role in metabolic pathways. Prokaryotic alpha-enolase may contribute to pathophysiological processes [28]. Additionally, surface-associated enolase is an adhesion-related factor in *M. bovis*, promoting adhesion by binding to plasminogen. Enolase could be a significant protein contributing to the virulence of *M. bovis* [29]. Although according to the available information, there are no reports comparing the difference in the prevalence of this virulence factor with other *M. bovis* virulence factors, the results of the current study revealed a higher frequency of the alpha-enolase gene among the selected virulence factors, indicating the particular functional importance of this protein.

The P48 protein is an immunodominant lipoprotein situated within the membrane. The P48 protein of *M. bovis* shares homology with the protein found in *M. agalactiae*. It serves as a valuable indicator of *M. bovis* infection and is an alternate candidate for the formulation of specific serological tests targeted at *M. bovis* [30]. Robino et al.[31] reported the detectability of the P48 protein in all the tested isolates. Fu et al.[32] developed a direct competitive ELISA for the detection of *Mycoplasma bovis* infection based on P48 protein.

Prysliak et al.[33] evaluated immune responses to ten proteins from *M. bovis*, including P48 and P81. These proteins demonstrated high conservation levels, exhibiting 98% to 100% similarities between the PG45, HB0801, Hubei-1, and CQ-W70 strains.

Consequently, these proteins emerge as promising targets for potential vaccines. Within the scope of this study, an exploration was undertaken to ascertain the frequency of select highly protected proteins, namely P48, P81, and LppB, in instances of bovine mastitis. Notably, P48 emerged as the second most prevalent virulence factor, with an incidence rate of 57.14%. Although there exists a lack of studies on the occurrence of this gene and other has chosen *M. bovis* genes, indications point toward the substantial significance of P48 [34]. LppB, an antigen shared among mycoplasmas, surfaced as a standard and distinct element [35]. In this study, the occurrence of this gene in samples collected from bovine mastitis cases registered at 52.38%, suggesting a possible pivotal role played by this gene in the pathogenicity of infections attributed to *M. bovis* in dairy cows. Encoded by the P81 virulence gene of *M. bovis*, the P81 protein stands as a membrane lipoprotein and represents one of the bacterial surface antigens. This specific protein has found utility in developing serological and PCR-based diagnostic methodologies for *M. bovis* [36]. A previous investigation spotlighted the efficacy of 9 recombinant proteins, including LppB and P81, when formulated with an adjuvant, in stimulating an effective Th17 response against *M. bovis*. Noteworthy results emerged from Zhang et al.[37] study, underscoring the inhibitory antibody titers found within the anti-P81 serum. Despite implementing several adjustments in the PCR method, the targeted gene could not be conclusively identified in this current study. However, it remains uncertain whether this gene is present in *Mycoplasma* strains within the study area, and ascertaining the abundance of this gene within the study population necessitates further research. Righter et al.[16] scrutinized 19 *M. bovis* isolates, discovering that using probes designed for *M. bovis* detection resulted in nucleotide disparities among 19 negative field samples. These disparities hindered probe hybridization, primarily due to critical discrepancies within the probe binding site. Across these isolates, the P81 gene encoding the membrane lipoprotein exhibited absence, truncation, or sequence elongation. This collection of findings signifies variations within the P81 gene that hinder primer or probe binding [36,37]. The study's results above partly clarify the current study's challenge in identifying *M. bovis* P81.

This current study unveiled the highest frequency of virulence genes within alpha-enolase and the lowest frequency within LppB. Notably, differences in the gene prevalence did not reach statistical significance, thereby underscoring the necessity for future research involving native isolates to definitively pinpoint the primary virulence factors.

Declarations

Availability of Data and Materials: The data that support the findings of this study are openly available.

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Ethical Approval: The procedures were applied in the method section were in accordance with the experimental local ethics committee (Code of ethics: IR.SKU.REC.1399.005).

Competing Interests: The authors have declared that no competing interests exist.

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

Author Contributions: Azam Moktari and Masoud Ghorbanpoor Najaf Abadi designed the study and performed analysis, Negar Ghazvineh performed the tests. Ali Kadivar collected the samples and performed the test in the field. Azam Mokhtari and Somayeh Shahrokh Shahraki wrote the manuscript. All authors read and revised the manuscript.

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RESEARCH ARTICLE

Recombinant Porcine Interferon Alpha Enhances the Humoral and Cellular Immune Responses to Porcine Transmissible Gastroenteritis Virus Inactivated Vaccine in Piglets

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Abstract

In this study, the effect of recombinant porcine interferon alpha (rPoIFNα) on porcine transmissible gastroenteritis virus inactivated vaccine (TGEV IV) in terms of immunological augmentation was examined. Seven experimental piglet groups, including PBS group, rPoIFNα group, inactivated vaccine (IV) alone group, $4.0x10^4$ U rPoIFNα+IV group, $2.0x10^5$ U rPoIFNα+IV group,$1.0x10^6$ U rPoIFNα+IV group, $5.0x10^6$ U rPoIFNα+IV group, were divided. The piglets in each group received a secondary vaccination at 28 days following the initial immunization. By using the ELISA assay, neutralization assay, MTT assay, and flow cytometry, we measured anti-TGEV-specific antibody expressions, neutralization antibodies, as well as lymphocyte proliferation index (Stimulation index, SI), specific IL-4 and IFN-γ production, and T cell subpopulations (CD3+, CD4+, and CD8+). Piglets injected with IV supplemented with rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U developed significantly higher anti-TGEV-specific and neutralizing antibodies compared to those treated with IV alone. IV therapy with rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U can boost cellular immunity against TGEV by increasing SI, IL-4, IFN-γ, and the ratio of CD3+, CD4+, and CD8+ cell subgroups. The IV+$5.0x10^6$ U rPoIFNα group showed a considerably larger immune increase than the IV+$2.0x10^5$ U rPoIFNα group, suggesting that it works in a dose-dependent manner. Therefore, rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U enhances the immune response against TGEV IV and may function as an immune stimulant.

Keywords: Cellular immune response, Humoral immune response, Inactivated Vaccine (IV), Porcine transmissible gastroenteritis virus (TGEV), Recombinant porcine interferon alpha (rPoIFNα)

Introduction

Porcine transmissible gastroenteritis (TGE), a highly contagious disease in pigs, is caused by the pathogen of the porcine transmissible gastroenteritis virus (TGEV) [1,2], with the exception of a non-pathogenic variant "porcine respiratory coronavirus (PRCV)" whose spike gene is deleted in TGEV and associated with mild or subclinical respiratory tract infections [3]. TGEV is classified as a member of the coronavirus family within the nidovirus order [4]. If piglets encounter this virus, it will cause severe diarrhea and a high mortality rate. The pig industry is at risk from this important virus [5].

Immunization has a high-quality capacity to prevent the TGE outbreak and TGEV infection. The development of the TGEV vaccine is crucial for the therapy and prevention of TGEV infection [6].

Currently, the efficiency of the TGEV-inactivated vaccination (IV) is insufficient [7]. Due to their high level

of safety, inactivated vaccines have been used extensively. However, they still have some drawbacks, such as short immunization duration and poor immunogenicity, the need for multiple booster doses, insufficient immunity in older animals, and strict production process safety regulations that drive up costs and cause unfavorable side effects [7-9]. Typically, adjuvants were needed in order for inactivated vaccines to be applied and have adequate immunogenicity [10]. Therefore, Boosting the TGEV vaccination's immune response is crucial for stopping and managing the spread of the TGE disease [11,12].

Various kinds of adjuvants have been employed to boost immunity and increase the efficacy of the TGEV IV vaccine, such as porcine interleukin-12 plasmid (IL-12 plasmid) and silicon nanoparticles (nano silicon particles) [13,14].

Studies on the adjuvant impact in the vaccination experiment have demonstrated that type alpha interferon, when administered parenterally with an inactivated vaccine formulation, has excellent adjuvant enhancement function [15].

According to studies, immune adjuvants like Freund's complete adjuvant and CpG use the interferon α of type I interferon as their main effector molecules. It significantly enhances the protective immunological response to influenza vaccines and animal foot-and-mouth disease (FMD) subunit vaccines, and leads those vaccinated individuals to develop large amounts of IgG2a antibodies [16,17].

According to research, when co-administered with an antigen, IFN-α/β can enhance the effects of a variety of immunological reagents, such as soluble proteins [18], inactivated vaccines [17], or recombinant DNA containing transgene materials [19].

Porcine interferon alpha (PoIFNα) has been demonstrated to possess cytokine (CK) properties and exhibit several fundamental biological roles. PoIFNα has long been the subject of research centered on its potent antiviral role, however, limited knowledge is known about its immunological function [20,21].

Previous studies have demonstrated that PoIFNα can generate an effective stimulating impact on porcine immune cells [22]. The recombinant porcine interferon alpha (rPoIFNα) prepared in *E. coli* has been demonstrated by way of us to inhibit the infection of TGEV *in vivo* [21] and *in vitro* [20].

We combined the soluble rPoIFNα made in our lab with the inactivated vaccine (IV) of TGEV in this study to further assess the viability of using recombinant porcine interferon in piglets [23]. We then co-injected them into pigs to explore the feasibility of developing a new mixed TGEV vaccine containing cytokine adjuvants.

Therefore, our research represents an *in vivo* examination of rPoIFNα's capacity to encourage the production of an immunological response to the TGEV-inactivated vaccine (IV) in pigs.

Through animal testing, This investigation aims to comprehend rPoIFNα's potential as an adjuvant alternative for TGEV IV. It also aims to offer a new option available for preventing piglets from contracting TGEV and reducing the incidence of TGE.

Material and Methods

Ethical Statement

The animal experiment project of this study was approved by Anhui Medical University's Bioethics Committee (Hefei, Anhui, People's Republic of China) (Approved Serial number. LLSC20180307, Approved date:01.03.2018). The authors also affirmed that they had followed with all applicable international requirements and standards regarding the use and care of laboratory animals for research purposes.

Reagents and Drugs

Experiments were conducted using Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) obtained from Sigma Co. Ltd. Fetal Bovine Serum (FBS) was procured from Gibco Corp, while the TGEV antibody detection package (ELISA kit) was obtained from Ingenasa Tech. Catalog serial number 11.TGE.K.3/2, manufactured by Ingenasa Tech in Madrid, Spain.

The TGEV IV oil emulsion, which served as the vaccine control group, was manufactured and procured from TECBOND Biological Products Co., Ltd., located in Chengdu, Sichuan, P.R. China.

The recombinant porcine interferon α Protein (rPoIFNα) was synthesized by Wuhu Interferon Bio-products Industry Research Institute Co., Ltd (Wuhu, Anhui, P.R. China) using the established research and development methodologies employed by our research group [23], it is frozen in an ultra-low temperature refrigerator at -80°C.

Immunization of Experimental Animals in Groups

In this study, 28 white SPF Landrace pigs that were 30 days old were used; they were grown and kept in the barrier facility of the Animal Experiment Center of Anhui Medical University. The pigs had been obtained from a commercial herd that no longer included TGEV. During the tests, they consumed food and liquids *ad-libitum* throughout the experiments.

ELISA tests and PCR tests have been used to screen and verify that all pigs did not comprise TGEV-specific antibodies and that all viral genomes of porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), pseudorabies virus (PRV), porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV) and TGEV were negative. This was done to ensure that the experimental piglets (participants) did not carry TGEV and different pig-related pathogens [24-26].

The inactivated vaccine of TGEV was prepared with the following procedures:

(1) A TGEV was inoculated in ST cells at a cell density of 80% at a dosage of 5 MOI. Subsequently, an adequate quantity of MEM medium supplemented with 5% FBS was added, followed by incubation at a temperature of 37°C in a 5% CO_2 incubator for a duration of 24-72 h. The culture supernatant was collected when the cell lesion rate was not less than 80%, and then the culture supernatant underwent a series of three freeze-thaw cycles, followed by centrifugation at 3.000 rpm for a duration of 10 min. When the $TCID_{50}$ value was not less than $10^{-6.0}$, and then formaldehyde and Binary ethylenimine diethylenimine (BEI) were added to inactivate the virus. The final concentration of BEI was 5 mmol/L and the final mass fraction of formaldehyde was 0.2%.

After sufficient mixing, the mixture was placed at the temperature of 30°C, shaking for 72 h for virus inactivation; finally, the final concentration of 5 mmol/L of sterile sodium thiosulphate solution was added to terminate the inactivation for 1 h, and then mixed with sterile Tween-80 at a mass ratio of 24:1 mixed to make the aqueous phase;

(2) White oil, Spencer-80, and aluminum stearate were mixed in mass fraction 48:2:1 and autoclave sterilize was performed to make the oil phase;

(3) The aqueous phase and the oil phase were homogenized and emulsified at 10.000 rpm in a mass ratio of 1:1 for 3 min each time, and repeated 3 times, and then content determination test, sterility test, mycoplasma test, and exogenous virus test were carried out. When the test results were in accordance with the Veterinary Pharmacopoeia of the People's Republic of China, the described inactivated porcine transmissible gastroenteritis vaccine IV was obtained, and the commonly used dosage of the vaccine was 1 mL/piglet.

The piglets were allocated into seven groups, with each group consisting of four piglets, using a random assignment method. Each piglet belonged to 30-day-old SPF Landrace white piglet. Except for the first blank PBS control group and the second rPoIFNα control group, each of the third to seventh groups was injected with a mixture of 1 mL of TGEV IV and 1 mL of different doses of rPoIFNα. After the mixture (the adjuvant and IV) is evenly mixed, we inject it into the pig. The first blank control group received 2 mL PBS as an immunization, the second rPoIFNα control group received 1 mL PBS+1 mL $2.0x10^5$ U rPoIFNα, the third vaccine control group received the 1 mL PBS+1 mL TGEV IV, and the fourth group received a combination of 1 mL $4.0x10^4$ U rPoIFNα+1 mL TGEV IV. 1 mL TGEV IV+1 mL $2.0x10^5$ U rPoIFNα were administered together to Group 5. 1 mL TGEV IV+1 mL $1.0x10^6$ U rPoIFNα were administered together to Group 6. 1 mL TGEV IV+1 mL $5.0×10^6$ U rPoIFNα were administered together to Group 7.

A total of 2 mL of each preparation was injected. The mass of piglets was measured and their body temperatures were documented at 8 a.m. on the test day. Then, in accordance with the experimental grouping strategy, intramuscular injections were delivered to each pig into its neck. All of the experimental pigs received a boost vaccination on the 28th day following their first immunization. Secondary immunization of each group of piglets was performed with a vaccine solution of whose composition and quality were the same as the first immunization of each group (PBS/rPoIFNα+IV). The study design and experimental grouping schemes are shown in *Fig.1*.

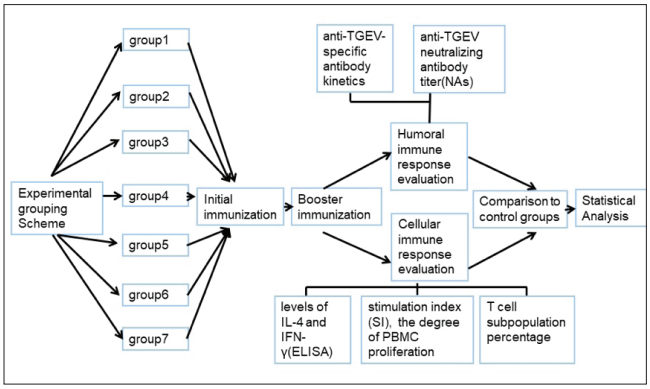

Fig 1. Study design and experimental grouping scheme

Clinical Symptom Tracking

(1) Clinical symptom recording: Following vaccination, the experimental pigs' mental state, hunger, respiratory health, skin color, and other clinical indicators were observed each day.

(2) Measurement of body temperature: After the experiment began, the pig's body temperature was recorded daily at 8 a.m. and 8 p.m., and the recording was kept up until the study's ending.

(3) Measurement of body weight: After the trial began, the piglets' body weights were assessed every six days at 8:00 a.m. (Daily Gain/Original Weight, RDWG, relative daily weight gain) was used to compute relative weight changes.

Pig Blood Sampling

Test tubes containing aseptic vacuum lithium heparin (150 USP units) were utilized to collect blood samples at specific time intervals: 1, 7, 14, 21, 28, 35, 42, and 56 days post-inoculation (dpi). Blood samples that were not subjected to anticoagulation were collected for the purpose of serum separation. The obtained sera were then kept at -20°C in a low-temperature refrigerator.

Separation of Peripheral Blood Mononuclear Cells (PBMC)

PBMCs were extracted from pig heparinized blood using a density gradient centrifugation technique, as previously reported [27,28].

Test for PBMC Proliferation Specific to TGEV

Cultured PBMC cells were stimulated using purified TGEV antigens. As a negative control, PBMC that had not been activated by the TGEV antigen was employed. Positive controls included PBMC activated with the mitogen ConA (10 g/mL).

The proliferation response of PBMC cells was assessed using the common MTT methodology. The stimulation index (SI) was employed to quantify the extent of peripheral blood mononuclear cell (PBMC) proliferation. It is calculated by dividing the average value of the stimulated data by the average value of the unstimulated control [28].

Measuring the Antibody Titers Against TGEV

An enzyme-linked immunosorbent assay (ELISA) kit (Catalog Serial No: 11.TGE.K.3/2, Ingenasa Tech, Madrid, Spain) was employed following the guidelines provided by the manufacturer to detect antibodies specific to TGEV in pig serum samples. The titer was reported using the S/P ratio [21,25,29]. The serum sample was deemed to be positive when the result was 0.4 or above, and it was deemed to be negative when the value was less than 0.4.

The Quantification of Serum TGEV Neutralizing Antibodies (Nas)

The serum neutralization (SN) assay was conducted in accordance with the previously reported experimental methodology [30]. Serial 1:2 dilutions of all pig serum samples were performed. The 200 $TCID_{50}$ TGEV virus suspension was then combined with the serial serum dilutions, incubated at 37°C for 1 h, and then transferred to a PK-15 cell monolayer in a 96-microwell cell culture plate. The CPE formed in each microwell was then examined following a 72-h incubation period. The TGEV-neutralizing antibody titer in each serum sample, reported as ND_{50}, was determined using the Reed-Muench formula [31] (The maximum dilution required to achieve a 50 percent neutralization of viral activity.).

Experiment to Determine Cytokines

Based on the expression patterns of the cytokines they release, Th cell subpopulations may be identified. Interleukin-2 (IL-2), Interferon-gamma (IFN-γ), and lymphotoxin are the primary products of Th1 cells, whereas IL-13, IL-10, IL-6, IL-5, and IL-4 are the main output products of Th2 cells. Following stimulation, the levels of peripheral blood lymphocyte proliferation and the release of interferon-alpha (IFN-γ) and interleukin-4 (IL-4) can serve as indicators of the progression of acquired immunity. Porcine IL-4/IFN-γ was measured using ELISA kits purchased from Adlitteram Diagnostic Laboratories Corporation. The sensitivity of the test was determined to be 1 pg/mL using Adlitteram Diagnostic Laboratories (ADL, San Diego, CA, USA) after following the instructions provided by the manufacturer. The measurement of optical density was conducted at a wavelength of 450 nm using an automated Bio-Rad Model 450 microplate ELISA reader manufactured by Bio-Rad in Hercules, CA, USA [32-35].

Peripheral Blood Lymphocyte Flow Cytometric Analysis

Resuspended PBMCs were supplemented with antibodies against the porcine CD molecule (anti-CD3ε-FITC, anti-CD4α-PE, and anti-CD8-SPRD) at a density of 1×10^7 cells/mL using different fluorescent dyes. The source of the antibodies is Southern Biotech, located in Birmingham, AL, USA. Using the flow cytometry technique, the frequency of CD_8^+, CD_4^+, and CD_3^+ immune cells was quantitatively examined (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

To conduct the statistical study, the researchers employed SPSS 17.0 Software (SPSS Inc., Chicago, IL, USA). The mean and standard deviation (SD) results of the experiment were provided. The t-test was employed to conduct a comparison between two groups, whereas the one-way analysis of variance (one-way ANOVA) was utilized to compare variables across many groups. If the P-value is less than 0.01, the observed differences are considered to be statistically significant.

Results

Each Experimental Pig was Not Infected with Swine-Related Viruses

Both the ELISA and the PCR screen test results confirmed that none of the pigs tested exhibited antibodies specific to the porcine transmissible gastroenteritis virus (TGEV). Additionally, the results indicate the absence of porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine parvovirus (PPV),

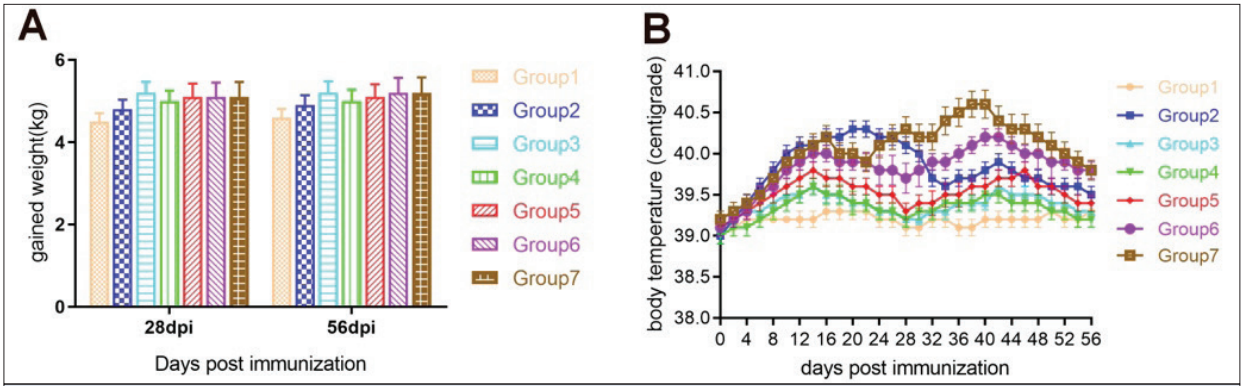

Fig 2. Piglets need to be monitored for clinical signs at various time points following initial and booster vaccinations. These monitors include, but are not limited to, recording the change in the average weight growth of the piglets (A) and the average rectal temperature change of the piglets (B). Body weight growth was recorded in kilograms, while fever was defined as when a piglet's rectal temperature reached or exceeded 41°C. The data are reported as mean±standard deviation (SD), with each group containing four piglets, respectively

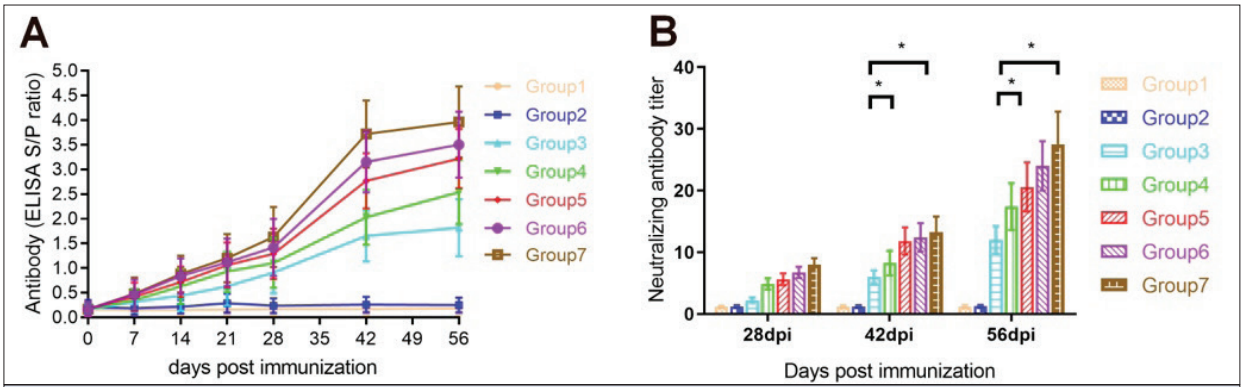

Fig 3. Levels of anti-TGEV specific antibodies (A) and titers of anti-TGEV neutralizing antibodies (B) in vaccinated experimental piglets at different times. According to the grouping scheme, seven groups of experimental piglets received intramuscular injections of different biochemical reagents, such as PBS alone, or IV alone, or rPoIFNα alone, or a combination of $4.0x10^4$U rPoIFNα+IV, or a combination of $2.0x10^5$U rPoIFNα+IV, or a combination of $1.0x10^6$ U rPoIFNα+IV, or a combination of $5.0x10^6$U rPoIFNα+IV. The levels of anti-TGEV specific antibodies and the titers of neutralizing antibodies in the blood samples from each animal group were assessed using the ELISA assay and the serum neutralization (SN) assay. The S/P ratios were employed to quantify the levels of anti-TGEV-specific antibodies, whereas the ND_{50} (The maximum dilution required to achieve a 50 percent neutralization of viral activity) form was utilized to measure the levels of anti-TGEV neutralizing antibodies. The outcomes were reported as the mean±SD (standard deviation). The asterisk (*) indicates a significant difference (P<0.01) between the two groups

porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV), or TGEV viral genomes. This measure was implemented to ensure the absence of TGEV and other pig-associated viruses in the piglets comprising the trial participants [24-26].

Clinical Signs in Each Pig Group Underwent Experimentally

Following the first inoculation and booster immunization, the pigs were weighed at various intervals.

Although there were little observed differences in the body weight growth (weight gain) of the piglets among the groups, the overall size of the piglets increased *(Fig. 2-A)*. Furthermore, none of the piglets in any group had a fever, and their rectal temperatures were all lower than 41°C *(Fig. 2-B)*. The aforementioned findings indicate that the vaccination administration did not provide any significant impact on the clinical performance of the experimental piglets in either of the groups.

Specific Anti-TGEV Antibody Titers

Fig. 3-A illustrates the kinetics of the fluctuating levels of TGEV-specific antibody responses in seven experimental groups, as assessed through the use of ELISA assay.

Pig groups inoculated with IV+$1.0x10^6$ U and IV+$5.0x10^6$ U rPoIFNα at 28 dpi exhibited significant antibody production against TGEV. Moreover, the production of these antibodies experienced a substantial increase subsequent to the booster immunization.

The IgG levels observed in the IV+$1.0x10^6$ U rPoIFNα group and the IV+$5.0x10^6$ U rPoIFNα group were found to be considerably elevated compared to the IV alone group at 42 dpi and 56 dpi (P<0.01).

At various points after vaccination, the IV+$1.0x10^6$ U rPoIFNα group and the IV+$5.0x10^6$ U rPoIFNα group's piglets showed greater antibody titers than the IV immunization alone group's. It is deduced that rPoIFNα

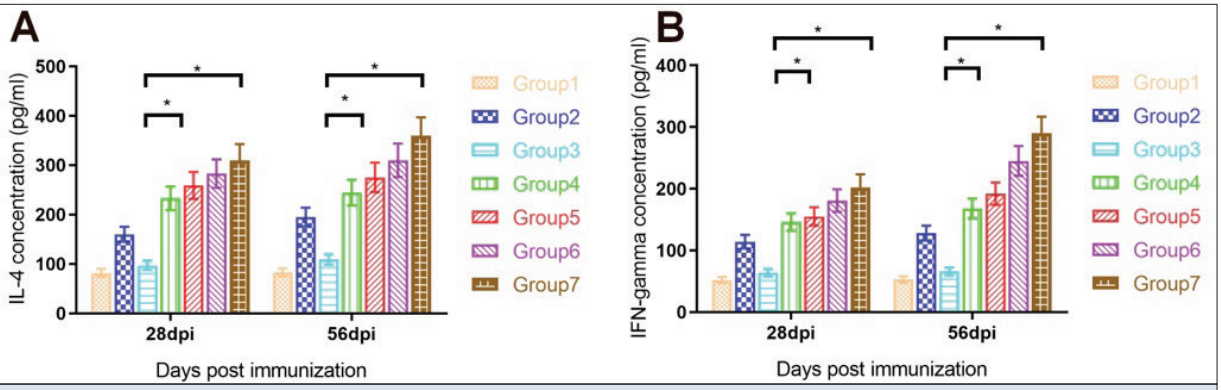

Fig 4. The concentrations of IL-4 (A) and IFN-γ (B) in the PBMC supernatant of experimental piglets at 28 and 56 dpi were measured using ELISA assays. The columns in the table present the average and variability (standard deviation) of the cytokine levels. These levels were measured in triplicate for a group of four subjects. The asterisk (*) indicates a significant difference (P<0.01) between the two groups

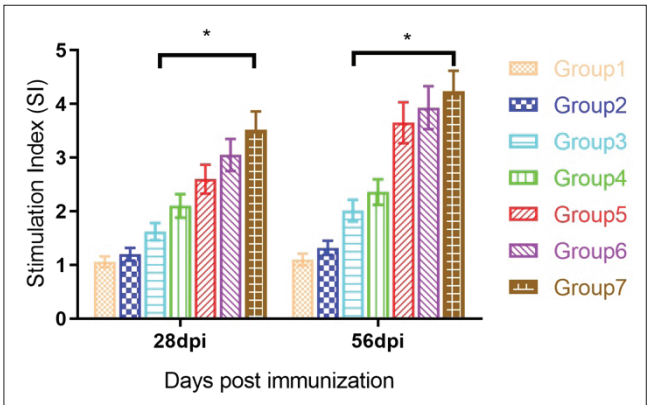

Fig 5. The values of the stimulation index (SI) represent the amounts of PBMC proliferation in each of the experimental groups. The group mean±SD is provided, and the SI value, which was determined in triplicate, is included in each column. ConA serves as a positive control, and the asterisk (*) indicates a statistically significant difference (P<0.01) between the two groups

probably works in a dose-dependent manner to increase antibody response.

Neutralizing Antibodies are Determined Using the SN Test

Overall, the neutralizing antibody titers from the IV+1.0x10^6 U rPoIFNα group and IV+5.0x10^6 U rPoIFNα group were considerably greater than those from the IV alone group at 28 dpi and 56 dpi *(Fig. 3-B)*.

Pigs inoculated with 2.0x10^5 U of rPoIFNα or PBS alone failed to produce any neutralizing antibodies against TGEV. Compared to the IV alone group, the IV+2.0x10^5 U rPoIFNα group was able to develop moderate higher amounts of neutralizing antibodies. Furthermore, the group treated with IV+1.0x10^6 U rPoIFNα and the group treated with IV+5.0x10^6 U rPoIFNα exhibited higher levels of neutralizing antibodies compared to the group treated with IV alone. This is indicative of an enhanced humoral immune response to TGEV.

Specific Cytokines Secreted in Porcine PBMC Supernatant After TGEV Antigen Stimulation

The production levels of IFN-γ and IL-4 in the supernatant of swine PBMC were assessed using commercially available ELISA kits. This evaluation was conducted after stimulating the PBMCs with TGEV antigen.

The findings of the study indicate that the co-administration of rPoIFNα and IV vaccine can effectively elevate the production levels of IL-4 and IFN-γ. These cytokines play a crucial role in stimulating Th1 and Th2 immune responses and augmenting cell-mediated immunity (CMI) in piglets at various time intervals following immunization *(Fig. 4-A,B)*. This is done when the piglets' peripheral blood lymphocytes are stimulated with the TGEV antigen.

RPoIFNα Induces Proliferation of the TGEV-Specific PBMCs (SI)

The rPoIFNα-mediated lymphocyte proliferation levels

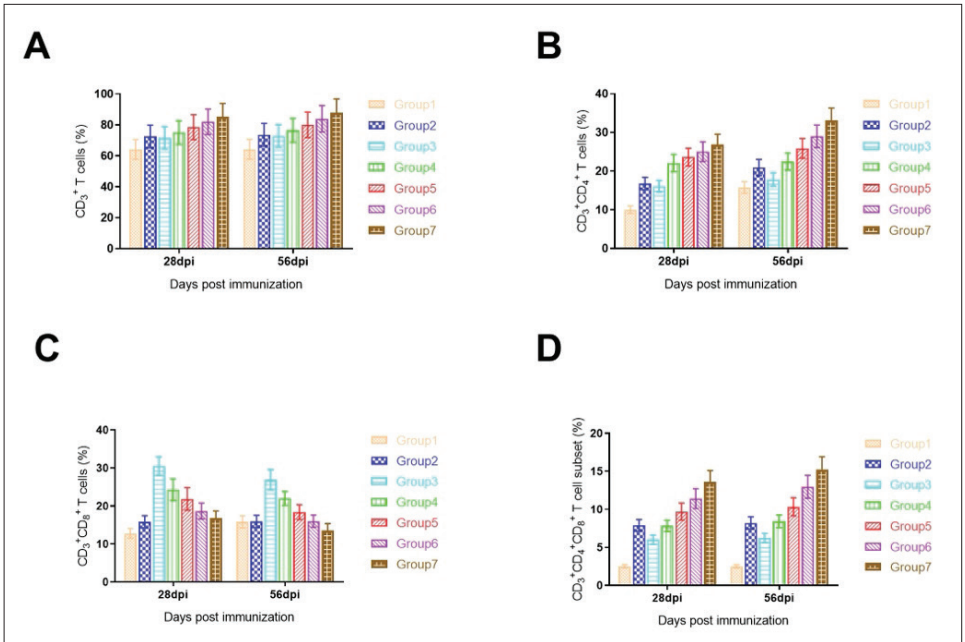

Fig 6. Distinct profiles of CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$, and $CD_3^+CD_4^+CD_8^+$ cells were documented after the initial vaccination and subsequent booster immunization procedures. Panel A exhibits the proportion of CD_3^+ cells; Panel B exhibits the proportion of $CD_3^+CD_4^+$ cells; Panel C exhibits the proportion of $CD_3^+CD_8^+$ cells; and Panel D exhibits the proportion of $CD_3^+CD_4^+CD_8^+$ cells

(expressed in stimulation index, SI) were assessed using the MTT colorimetric test *(Fig. 5)*. The piglet cells from the IV+1.0x10^6 U rPoIFNα group and the IV+5.0x10^6 U rPoIFNα group produced higher stimulation index (SI) at 28dpi, respectively, in comparison to the group receiving PBS alone and the group receiving IV alone. The statistical analysis revealed significant differences among the groups ($P<0.01$). Specifically, the SI of the IV+5.0x10^6 U rPoIFNα group exhibited the greatest level at 56 dpi ($P<0.01$) in comparison to both the PBS-alone group and the IV-alone group. Based on the established association between PBMC proliferative response and cell-mediated immune response (CMI), the findings of this study indicate that the inclusion of rPoIFNα enhances the CMI of the IV administration in piglets.

Flow Cytometry Analysis of T Cell Subpopulations in Piglets' Blood Specimens.

The evolving profiles of the CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$, and $CD_3^+CD_4^+CD_8^+$ T cell subpopulations are shown in *Fig. 6*. Early in the post-first vaccination interval, $CD_3^+CD_8^+$ and $CD_3^+CD_4^+CD_8^+$ T cell counts increased somewhat in the majority of samples. At 56 dpi, Piglets' blood showed a notable increase in their values following booster vaccination. Among groups, differences were statistically significant ($P<0.01$). At 28 dpi, these growth values' arguments commenced.

Discussion

Numerous investigations in recent years have focused on examining the impact of IFNα as adjuvants in prophylactic vaccinations. In 2006, de Avila Botton et al.[16] co-injected porcine IFN-α with the adenovirus containing the A24 capsid of the FMDV and the 3C enzymatic protease encoding region (Ad5-A24) into the pigs, the result could offer full protection, but the control group with only adenovirus could only postpone the development of viremia.

Similar to this, Cheng et al.[36] cloned the swine interferon alpha gene into the pcDNA3.1 vector, created a recombinant vector, and administered it to pigs together with the protein vaccine for porcine FMD. This led to the induction of many neutralizing antibodies that are specific for FMD as well as a robust immunological response driven by T cells. However, the adjuvant-free control group can only stimulate a minimal cellular and humoral immune response [36]. It was also shown that interferon, when used as an adjuvant for protein vaccines, was able to stimulate a significant amount of inflammatory cytokines *in vitro* and activate a Th1-type immune response.

The pVAX1-gag-gp120 (DNA vaccine plasmid against HIV) and the pVAX1-IFN (eukaryotic expression plasmid) were both built by Jiang et al.[37] based on the eukaryotic expression vector pVAX1. The BALB/C mice were given an intramuscular injection with the mixed resulting vaccine. In the mouse spleen T cells, according to the findings in comparison to the control group without adjuvant, there were substantial increases in the percentages of the lymphocyte $CD_3^+CD_8^+$ and $CD_3^+CD_4^+$

subpopulations as well as the cytotoxic activity of spleen cytotoxic T cells [37].

Proietti et al.[17] revealed that endogenous type I interferon in mice was required for the Th1 type immune response elicited by conventional adjuvants. Interferon also showed strong adjuvant efficacy when paired with human influenza vaccination owing to the development of IgG2a and IgA.

In support of this claim, Bracci et al.[38] showed that IFN-α may have a positive adjuvant effect when the influenza vaccination is administered intramuscularly or mucosally.

IFN-α may also greatly boost the cellular immune response to the PRRSV vaccination, according to Charerntantanakul [22], who was searching for an appropriate immunological adjuvant for the pig PRRSV vaccine. Primary lymphocyte proliferation, cytotoxic T cell activity, peripheral blood lymphocyte IFN-γ production, and T cell reactivity to the PRRSV antigen are all markedly enhanced. Intriguing studies on the adjuvant impact of IFN-α on preventative vaccinations have also been published by de Avila Botton et al.[16], Le Bon et al.[18], and O'Brien et al.[39].

The development of neutralizing antibodies (NA) is a crucial component of humoral immunity, as it aids in the elimination of TGEV and the prevention of viral infections [6]. Within the context of the host immune response against TGEV infection, cell-mediated immunity (CMI) is also an essential component [21]. However, protective immunity against infection with heterologous TGEV strains may require a dual enhancement of CMI and antibody production [7]. These strongly indicate that the production of TGEV NA antibodies and virus-specific cellular immune responses collaborate to facilitate the elimination of the virus. This suggests that novel formulations of non-infectious vaccines and delivery systems are required to stimulate both humoral and cellular responses in order to effectively control TGE [7,11-14,21,35].

Our results indicate that the combination of rPoIFNα with the TGEV IV vaccine has the ability to enhance the immunological response. Our study specifically showed that when rPoIFNα was given along with an inactivated TGEV vaccination, it resulted in the activation of both Th1 and Th2 immune responses in pigs. rPoIFNα exhibits a highly favorable safety profile. The clinical signs of the pigs, such as weight increase and body temperature, were not impacted after immunization *(Fig. 2)*. After immunization, we also determined changes in parameters such as anti-TGEV-specific antibodies, NAs, cytokines IFN-γ and IL-4, lymphocyte proliferative activity (SI), and CD_3^+, CD_4^+, and CD_8^+ cell subpopulations.

The results showed the following findings:

(1) Regarding the TGEV-specific antibody titers, the rPoIFNα+IV group exhibited an earlier generation of TGEV-specific antibodies compared to the IV alone group. The group receiving both rPoIFNα and IV showed elevated levels of TGEV-specific antibodies compared to the group receiving IV alone. This indicates that rPoIFNα increases the antibody response specific to TGEV that is stimulated by the IV vaccine *(Fig 3-A)*.

(2) Regarding the levels of neutralizing antibodies, pigs vaccinated with rPoIFNα+IV showed a significant difference compared to those vaccinated with IV alone. This suggests that rPoIFNα boosts the production of neutralizing antibodies, which in turn may play a role in enhancing humoral immunity and providing protection against TGEV *(Fig 3-B)*.

(3) Th2 cytokines, such as IL-4, play a crucial role in boosting the humoral immune system, while Th1 cytokines, such as IFN-γ, mainly affect cell-mediated immune responses [40]. Regarding cytokines, the inclusion of rPoIFNα notably augmented the synthesis of IFN-γ and IL-4, indicating that rPoIFNα enhances both Th1 and Th2 reactions. This suggests that rPoIFNα enhances the efficacy of the TGEV vaccine and acts as an adjuvant for both the cellular and humoral immune responses to TGEV IV vaccination *(Fig 4)*.

(4) Regarding lymphocyte proliferative activities, our data demonstrated that the TGEV-specific lymphocyte proliferative response was considerably greater in the group that received the IV+rPoIFNα combination immunization compared to the group that received IV alone immunization ($P<0.05$) *(Fig. 5)*. Given that lymphocyte proliferative responses are typically linked to cell-mediated immunity (CMI), this implies that the administration of both IFN-α and IV can enhance the CMI of the TGEV IV vaccine.

(5) Regarding T cell subpopulations, we conducted a more detailed analysis of alterations in the subpopulations of CD_3^+, CD_4^+, and CD_8^+ cells using flow cytometry *(Fig. 6)*. During the early stage following the initial immunization, there was a small rise in the populations of CD_3^+, CD_4^+, and CD_8^+ cells in most groups. The quantities of CD_3^+ and CD_4^+ cells in pig blood exhibited a progressive increase in all groups at 28 dpi, followed by a considerable increase at 56 dpi subsequent to booster immunization.

We also found that the administration of rPoIFNα at dosages of $1.0x10^6$ U or $5.0x10^6$ U effectively boosted the immunological response of piglets when combined with the TGEV IV vaccination. More precisely, the inclusion of rPoIFNα in the TGEV IV vaccine resulted in improved

immune responses, both in terms of antibodies and cellular immune responses, when compared to the group that received only the TGEV IV vaccine. The results of our study indicated that the immune-boosting impact of rPoIFNα was more pronounced when administered at a dosage of $5.0x10^6$ U compared to a dosage of $1.0x10^6$ U. This suggests that the immunological response of piglets is enhanced by rPoIFNα in a dose-dependent manner. The long-lasting impact was most pronounced at a concentration of $5.0x10^6$ U. The results of our study align with the findings published by Tovey et al.[15] and Damjanovska et al.[41]. They noticed a considerable increase in IgG and IgA levels in bodily fluids when IFN-α was provided with inactivated influenza vaccine, and this increase was dependent on the dosage.

Our study showed that rPoIFNα exhibited good safety. After co-administration to piglets, the inactivated vaccine (IV) mixture including rPoIFNα may considerably increase the generation of antibodies, Th1 and Th2 cytokines, and PBMC proliferation when compared to TGEV IV alone. The piglets' immune response to TGEV was greatly enhanced by the combined administration. Furthermore, when the dose of rPoIFNα was increased, the immunological boost induced by the IV+$5.0x10^6$ U rPoIFNα group was significantly bigger than that of the IV+$2.0x10^5$ U rPoIFNα group.

Thus, the addition of rPoIFNα at doses of $1.0x10^6$ U or $5.0x10^6$ U to the IV vaccine resulted in higher levels of antibody titers, lymphocyte stimulation index (SI), IFN-γ and IL-4 secretion, and lymphocyte subpopulations compared to immunization with the TGEV IV vaccine alone. To our knowledge, this work represents the first investigation conducted in a living organism to assess the ability of rPoIFNα to enhance the immune response to the TGEV IV vaccine in piglets. The aforementioned results indicate that rPoIFNα has the potential to serve as an adjuvant, augmenting the immunological response to vaccines in farm animals.

Limitations of This Study: This study aimed to assess the suitability of rPoIFNα as an adjuvant for immune activation in vaccinations for farm animals. Nevertheless, additional field challenge tests are required to assess and thoroughly analyze the protective efficacy of rPoIFNa+IV on piglets.

In summary, rPoIFNα showed significant adjuvant effects when combined with TGEV- inactivated vaccines at doses of $1.0x10^6$ U or $5.0x10^6$ U. The results of our study show that rPoIFNα is an ideal adjuvant for immunostimulation of TGEV. The significance of our findings lies in the revelation of rPoIFNα's potential as an optimal immunostimulatory adjuvant for TGEV-inactivated vaccines.

Declarations

Availability of Data and Materials: The authors affirm that they can provide the data that supports the findings of this study if a legitimate request is made.

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Competing Interests: The authors declared that there is no conflict of interest.

Ethical Approval: The animal experiment project of this study was approved by Anhui Medical University's Bioethics Committee (Hefei, Anhui, People's Republic of China). (Approved Serial number. LLSC20180307, Approved date:01.03.2018).

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

Author Contributions: HYY: Conducted data inquiry, performed data analysis, and drafted the manuscript. DMG: Data acquisition, investigation, and methodology. JD: Conducted data analysis and performed experiments and methods. YS: focused on designing the study, conducting thorough data analysis, and providing critical feedback for revising the manuscript. JZ: Engage in the examination, creation, oversight, and revision of the manuscript. All authors revised and approved the final version of the manuscript.

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Research Article

Effects of Electroacupuncture on Behavioral deficits, Hippocampal Neuronal Death and Oxidative Stress in Rats with Parkinson's Disease

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Abstract

We aimed to study electroacupuncture (EA) for its potential neuroprotection against oxidative stress, death of hippocampal neurons and behavioral deficits in rats with Parkinson's disease (PD). PD was induced by rotenone (2 mg/kg, subcutaneous injection) in male Wistar rats. EA group, control group, miR-331-5p antagomir group, model group and EA + miR-331-5p antagomir group were set up. The novel object recognition tests together with shuttle box tests were conducted to examine the cognitive function, and the pain sensitivity was measured by the tail flick test. In comparison to the control group, the cognitive function and thermal pain threshold significantly declined in the model group. The model group had decreased superoxide dismutase (SOD) and superoxide dismutase (SOD) activities, decreased brain-derived neurotrophic factor (BDNF) expression and increased malondialdehyde (MDA) level in the hippocampus. More hippocampal neuronal loss was detected from the model group. EA increased the hippocampal GPx plus SOD activity and decreased the MDA content. EA or miR-331-5p antagomir raised the quantity of alive hippocampal neurons and the BDNF expression. EA can prevent rotenone-induced non-motor disorders through antioxidation and neuroprotective functions.

Keywords: Electroacupuncture, miR, Neuroprotection, Oxidative stress, Parkinson's disease

Introduction

Belonging to a multi-system neurodegenerative illness, Parkinson's disease (PD) manifests motor or non-motor symptoms (NMSs) as the features. Known as a most frequently occurring NMS in PD patients, cognitive impairment results from neuronal injury in memory-related cerebral regions such as the hippocampus [1]. Studies have been extensively carried out on the hippocampal function and structure in PD sufferers, with the hippocampal volume decrease accompanied by cognitive impairment reported in some cases [2]. With an estimated prevalence of 68-95%, PD is characterized by pain as an additional common NMS, reducing patients' quality of life [2]. Pain in PD has a multifactorial etiology, and there has been no proper treatment yet at present.

Electroacupuncture (EA) is a noninvasive complementary therapy with application to relieve PD symptoms for a long time [3]. Through the normalization of basal ganglia neurotransmitters [4,5], EA stimulation at a high frequency (100 Hz) suppresses neuroinflammatory responses [6], alleviates oxidative stress as well as increases neurotrophic factor levels [7,8], thereby improving the movement function of diversified PD animal models. However, the exact mechanism of EA in relieving the motor symptoms of PD has not been clearly explained. For PD, miR-331-5p, as an exosomal miRNA, has become a potential biomarker. Its elevated expression may play a crucial role in the pathological progression of PD [9,10]. However, no reports are available on the role or function of miR-331-5p in the progression of PD.

Antagomir is an oligonucleotide that specifically inhibits the function of miRNA and can regulate the expression of target genes by binding and suppressing miRNA activity [11]. Therefore, we intended to test the role of miR-331-5p antagomir or EA in the progression of rotenone-induced PD in rats from the aspects of oxidative stress, neural damage and neural function. Additionally, EA has never been combined with miR-331-5p inhibition in the context of PD until now. This study may thus provide a novel strategy for addressing the multifaceted pathophysiology of PD.

Material and Methods

Ethical Approval

This study has been approved by the animal ethics committee of our hospital on June 28th, 2022 (Approval No. CMC202206003), and great efforts have been made to minimize the animals' suffering.

Reagents

Dimethyl sulfoxide (DMSO), ethanol and xylene sourced from Merck (Germany). Rotenone was provided by Sigma (USA). ELISA kit for brain-derived neurotrophic factor (BDNF) was bought from Wuhan Boster Biological Technology Co., Ltd. (China).

Laboratory Animals

The Laboratory Animal Research Center of Cangzhou Medical College (China) supplied male Wistar rats (n=70, SPF grade, 200-220 g) for breeding with a 12/12 h dark/light cycle, controlled humidity (50-55%) and temperature (22±2°C) as well as freely offered water plus standard food.

Experimental Design

An EA group, a miR-331-5p antagomir group, a model group, an EA + miR-331-5p antagomir group, and a control group were set (n=14). Seven rats of each group were used for inhibitory passive avoidance memory test, novel object recognition test and tail flick test. The remaining seven rats of each group were sacrificed for hippocampus-related experiments. Subcutaneous injection for 5 consecutive weeks with 98% sunflower oil plus 2% DMSO (1 mL/kg) was performed on rats in the control group once daily. In the model group, the rats were daily given 2 mg/kg rotenone (5 consecutive weeks of subcutaneous injection) once. In the EA group, the rats received 5 consecutive weeks of EA stimulation (100 Hz) at 1 h before subcutaneous injection of 2 mg/kg rotenone, once daily. Briefly, the acupoints Baihui (GV20, located at the midpoint of the line connecting the apexes of both ears) and Dazhui (GV14, located just underneath the cervical spinous process) were separately punctured using a stainless-steel EA needle (diameter: 0.25 mm) by 5 mm in depth. Next, a Han's acupoint nerve stimulator (HANS, Neuroscience Research Institute, Peking University) was employed to produce bi-directional square-wave electrical pulses (duration: 0.2 ms, 100 Hz), which were delivered for 30 min per day to the rats for 5 consecutive weeks (6 days weekly). The stimulation intensity gradually rose to 3 mA from 1 mA to 2 mA, which lasted for 10 min per time. The rats remained under an awake and unrestricted state in cages during EA. In the miR-331-5p antagomir group, at 1 h prior to 2 mg/kg rotenone subcutaneous injection, 10 mg/kg miR-331-5p antagomir was subcutaneously administered once daily to the rats for 5 consecutive weeks. In the EA + miR-331-5p antagomir group, at 1 h previous to 2 mg/kg rotenone injected subcutaneously, the rats received 5 consecutive weeks of EA stimulation (100 Hz) and miR-331-5p antagomir (10 mg/kg, subcutaneous injection) once daily.

For the purpose of PD induction in rats, rotenone at 10 mg/mL in concentration was prepared by means of dissolution in DMSO first and then dilution using sunflower oil (98% sunflower oil plus 2% DMSO), which was once daily administrated at 2 mg/kg *via* subcutaneous injection for 5 weeks. The preparation of fresh solution was conducted at an interval of 3-4 days [12]. The rats from the control group merely received DMSO together with sunflower oil, and the death rate was zero.

Inhibitory Passive Avoidance Memory Test

The rats were assessed by passive avoidance memory test on the passive avoidance memory using the shuttle box apparatus manufactured by Borj Sanat Azma (Teheran, Iran). The apparatus consisted of brightly illuminated and dark chambers of the identical size (40x40x30 cm), a stainless-steel bar floor (diameter: 2.5 mm, and spacing: 1 cm) in connection with an electric shock generator, and an opaque guillotine door (7x9 cm) utilized to separate the chambers. 24 h before the test, each rat was habituated to the apparatus for 5 min. There were two sessions (training and testing) completed in two days in a row. Specifically, the brightly illuminated chamber equipped with an elevated guillotine door was applied to accommodate the rat that was permitted to access the dark chamber during the training session. Then with the guillotine door installed therein dropped, the rat was subjected to 3 s of constant current shock (50 Hz, 1 mA). Finally, the rat was placed back to the home cage after being taken out from the dark chamber 30 s later. After 120 s, the aforementioned steps were repeated again. The training was stopped in the case of 2-min residence of the rat in the brightly illuminated chamber, and successful passive avoidance learning was recorded. In contrast, the shock was applied in the same way again when the rat went back to the dark chamber within 2 min. Each rat received training three times at most. Twenty-four hours after training, the memory retention test was performed without applying electric shock. The rat entered the brightly illuminated chamber again, and step-through latency (300 s at most) was acquired by recording the latency of passing through the guillotine door [13].

NOR Test

A black cubic box (60×60×20 cm) was used as an open field to carry out the NOR test. Specifically, the rat located in the box was allowed for 5 min exploration of two exactly the same objects on the first day. 24 h later, the rat entered the box containing the two familiar objects

again, one of which was substituted using a "novel" object. The rat explored the novel object and familiar object for 5 min, with the time for probing the two different objects recorded. The duration from the rat sniffing the object or staying within 2 cm surrounding the object was determined as the object exploration time. Data were calculated based on the formula below [14]: (time of probing the novel object/[time of probing the novel object + time of probing the familiar object] x 100%).

Tail Flick Test

A tail flick apparatus provided by Borj Sanat Azma (Iran) was adopted for the tail flick test to measure rats' pain threshold. Briefly, the rat was released in a Plexiglas restrainer, with its tail fixed in a groove below the radiant heat source. Tail flick latency was defined as the latency to withdraw the tail from the light beam. As for the setting of light intensity, the baseline brightness of 4.5-5.5 s was provided for the whole animals through illuminating on a light spot at 8 cm away from the tail tip. Tissue injury was minimized by a cut-off period lasting for 10 s. Tail withdrawal time of each rat was recorded three times, with an interval of 5 min and the average value obtained [13].

Sample Acquisition and Processing

When the behavioral tests were completed, the rats had a deep and permanent anesthesia before being quickly decapitated in a non-stressful environment. The hippocampal tissue was extracted from the brains for homogenization using ice-cold phosphate-buffered saline (PBS, 0.1 M) after being harvested on ice. After that, the homogenate was subjected to 10-min centrifugation (4°C and 3000 rpm). The resulting supernatant was aspirated and then preserved at -80°C for later biochemical assays.

Malondialdehyde (MDA) Content Detection

MDA content in the hippocampus was measured for lipid peroxidation quantification. Specifically, hippocampal samples (50 µL) were mixed with thiobarbituric acid (6%, 100 µL) and trichloroacetic acid (20%, 150 µL) prior to incubation at 95°C for 20 min in a boiling water bath. Sample centrifugation at 5000 rpm was executed for 5 min subsequent to cooling on ice. Next, a clean microplate was utilized to transfer the clarified supernatants in a volume of 200 µL, and the 535 nm wavelength was examined by a spectrophotometer (Bio-Tek, USA) to obtain the absorbance. Finally, MDA level was calculated as nmol/mg protein according to the 1,1,3,3-tetraethoxypropane-prepared standard curve.

Activity Determination of Glutathione Peroxidase (GPx)

The RANSEL assay kit (Randox Laboratories Ltd., UK) was applied to detect the GPx activity (in U/mg protein).

Activity Assay for Superoxide Dismutase (SOD)

The SOD activity was measured using the Naoyuki Taniguchi's method according to the capability of SOD to repress the reduction of nitroblue tetrazolium (NBT) dye triggered by superoxide anions resulting from hydroxylamine hydrochloride autoxidation. A reaction mixture containing NBT (24 µM), sodium carbonate (50 mM, pH 10.2), Triton X-100 (0.03%, 30 µL), and EDTA (0.1 mM) was prepared in order to assess the SOD activity. The reaction was started in a brain homogenate (10 µL)-containing cuvette added with the reaction mixture and hydroxylamine hydrochloride (1 mM). The absorbance at 560 nm was measured to calculate the reduction rate of NBT complex, with the results expressed as IU/mg protein.

Determination of BDNF Level

The mouse BDNF PicoKine™ double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Boster Biological Technology, Ltd., China) was used to measure the BDNF level in the hippocampus (pg/mg protein [15]). A multifunctional microplate reader (Bio-Tek, USA) was applied to test the 450 nm wavelength to acquire the absorbance.

Histological Analysis

Brain neurons were subjected to the cresyl violet staining for histological assays and to quantify neuronal injury. In brief, the rats were given Nesdonal overdose (100 mg/kg) to induce anesthesia. They were subsequently transcardially perfused with 0.9% saline and then 4% paraformaldehyde in a volume of 50 mL and 200 mL, respectively. Later, brains were immediately removed for overnight fixation with the same fixative. After dehydration using gradient alcohol, brain samples were subjected to xylene transparentization plus paraffin embedding. Afterwards, a cryostat (Leica, Germany) was utilized to prepare paraffin-embedded samples into 5-µm sections in the coronal plane. The serial brain sections underwent 0.1% cresyl violet staining following translocation to slides. Then a light microscope (BX-50 F, Olympus, Japan) for observation (40x) and a digital camera for photography were used for the sections. The hippocampal CA1, CA3, and dentate gyrus (DG) regions were selected for manual counting of Nissl-stained cells in three fields of each section. The average of three measurements from every region was taken as the final value.

Statistical Analysis

Statistical analysis and plot drawing were accomplished by GraphPad Prism 8.0 software (GraphPad Software, USA). Data were described by mean ± standard error of the mean (SEM). Comparisons among groups were performed through one-way analysis of variance, with

the Tukey's post-hoc test for analysis. P<0.05 denoted a difference with statistical significance.

Results

Passive Avoidance Test Results

The initial latency showed insignificant differences among the control group, model group, EA group, miR-331-5p antagomir group and EA+miR-331-5p antagomir group (4.32±0.61, 4.52±0.76, 3.81±0.69, 3.78±0.53 and 3.56±0.98 s) (P>0.05). The model group presented significantly shortened step-through latency by contrast to the control group (P<0.05), as well as to the EA and miR-331-5p antagomir groups (P<0.05), whereas it was significantly increased in the EA + miR-331-5p antagomir group in comparison to that in the model group (P<0.01) *(Table 1)*.

NOR Test (Learning Memory) Results

Compared to the model group, significant increases in DI were observed in the control group (P<0.05), together with in the EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group (P<0.01) *(Table 2)*.

Tail Flick Test Results

The model group exhibited a significantly shorter tail flick latency than the control group (P<0.05) and the EA, miR-331-5p antagomir and EA + miR-331-5p antagomir groups (P<0.01, P<0.05, P<0.01) *(Table 3)*.

Oxidative Stress in the Hippocampus

The model group, compared with the control group, had a significantly higher MDA level and significantly lower SOD and GPx activities (MDA: P<0.05, SOD: P<0.05, GPx: P<0.05). The EA, miR-331-5p antagomir and EA + miR-331-5p antagomir groups displayed a significantly reduced MDA level along with significantly enhanced SOD and GPx activities compared to the model group (MDA: P<0.05, SOD: P<0.05, GPx: P<0.05) *(Table 4)*.

BDNF Level in the Hippocampus

In comparison to that in model group, the BDNF level in the hippocampus was significantly elevated (P<0.01), and climbed in the EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group (P<0.05, P<0.05, P<0.01) *(Table 5)*.

Table 1. *Step-through latency (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
Step-through latency (s)	239.31±20.17	47. 22±7.36*	98. 14±8.62#	101.28±10.52#	150.58±11.98##

**P<0.05 vs. control group, *P<0.05, **P<0.01 vs. model group*

Table 2. *DI values (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
DI (%)	78.12±6.16	44. 25±4.31***	62. 12±8.29#	64.26±10.31#	76.21±8.21#

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 3. *Tail flick latency (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
Tail flick latency (s)	4.43±0.48	2. 94±0.31*	6. 06±0.58#	6.22±0.63#	8.25±0.53##

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 4. *Oxidative stress levels in the hippocampus (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
MDA (nmol/mg)	1.68±0.15	5. 21±0.62*	3. 04±0.58#	2.98±0.61#	2.25±0.31#
GPx (U/mg)	64.43±7.14	28.64±3.12*	48.98±6.15#	46.05±6.27#	69.68±7.28#
SOD (U/mg)	60.23±7.19	28.64±3.11*	39.68±4.15#	41.23±4.21#	59.02±7.18#

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 5. BDNF level in the hippocampus (n=7)					
Indicator	**Control Group**	**Model Group**	**EA Group**	**MiR-331-5p Antagomir Group**	**EA + MiR-331-5p Antagomir Group**
BDNF (pg/mg)	68.27±7.12	32.63±4.14**	48.62±4.12#	51.26±5.21#	71.13±8.39##
*P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group					

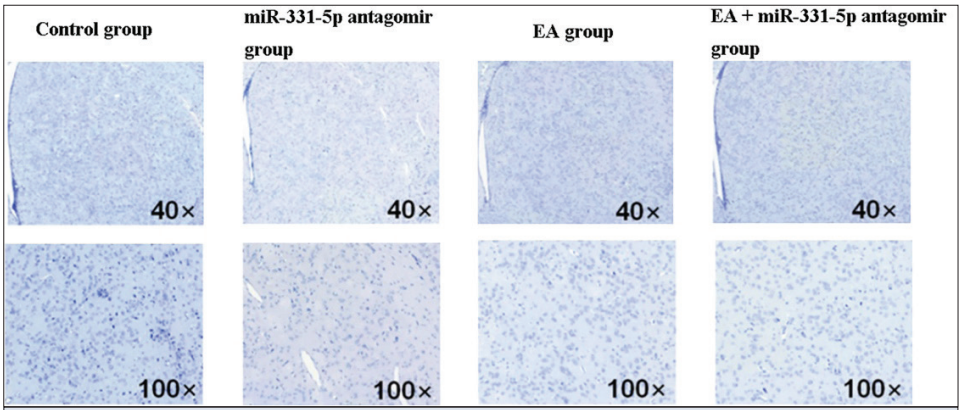

Fig 1. Light microscopic images for histological changes of sections of hippocampal CA1, CA2, CA3 and DG regions subjected to cresyl violet staining

Histological Changes of the Hippocampus

Cresyl violet staining was performed on the histological changes of the hippocampus. The results showed that compared with those in the control group, EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group, significant drops in Nissl-stained cell counts in the hippocampal CA1, CA2, CA3, and DG regions were observed in the model group *(Fig. 1)*.

Discussion

As a complementary and alternative therapy with the most extensive application, EA has been adopted to treat PD for a long period of time [16]. As revealed by increasingly more basic studies, EA is capable of alleviating central (vascular dementia, animal models of ischemic stroke, PD, Alzheimer's disease, spinal cord injury, traumatic brain injury, *etc.*) and peripheral (such as post-surgical injury or lipopolysaccharide injection) neuroinflammation [17]. For example, Cai et al.[18] reported that EA reduced cognitive impairment through anti-neuroinflammation in PD animal models. EA ameliorates intestinal motility disorders by regulating the 5-HT4R-mediated cAMP/PKA signaling in Thy1-αSyn mice with PD [19]. In addition, EA has protective effects on dopaminergic neurons from the murine substantia nigra of PD model, which may be related to its regulatory effects on ferroptosis-induced oxidative stress and apoptosis [20]. The present research was designed to probe into the neuroprotective role of EA in rotenone-induced cognitive impairment and neuronal injury. The results revealed that EA with antioxidant properties could efficiently relieve rotenone-triggered non-motor deficits plus neuronal injury. Hence, antioxidants may become a useful therapeutic approach for rotenone toxicity by lowering neuroinflammation and oxidative stress in the neurons.

One of the predominant symptoms in the rotenone-induced PD model is motor impairment. Rotenone neurotoxicity is associated with non-motor deficiencies involving depression, sleep disorders, and cognitive impairment in addition to motor dysfunctions [21,22]. Current studies have revealed through assessment by the passive avoidance and NOR tests that rotenone can induce memory impairment. This study yielded identical findings to earlier findings that rotenone exposure impairs memory *via* disruption of the blood brain barrier (BBB) mediated by microglia [23]. According to these research, oxidative stress created by rotenone disrupts the BBB and causes neuroinflammation, neuronal damage, and cognitive deficits [24]. In this study, it was found that the 5-week administration of EA alone or EA plus miR-331-5p antagomir could prevent memory impairment in the passive avoidance memory test. The NOR test showed that EA also relieved rotenone-induced cognitive disorder by increasing DI. As consistently denoted in reports, EA can mitigate amyloid-beta pathology and cognitive impairment in Alzheimer's disease by a novel mechanism involving activation of transcription factor EB [25]. Geng et al.[26] found that EA took advantage of the TRPC1 and SIRT1/AMPK signaling pathways to ameliorate mitochondrial dysfunction besides neuronal injury in PD mice. Therefore, EA becomes a crucial participant in preventing cognitive impairment. EA's ability to reduce rotenone-induced neuroinflammation may possibly be

responsible for its memory-enhancing properties due to its antioxidant effects.

With 40-85% of patients experiencing pain, it serves as a most ubiquitous NMSs in PD [27]. Research has indicated that malfunction in the mitochondrial electron transport chain (ETC) increases free radicals together with oxidative stress to participate in neuropathy [28]. This study demonstrated *via* the tail flick test that rotenone, a recognized inhibitor of mitochondrial complex I, enhanced the thermal pain sensitivity. These results demonstrated that various forms of pain are produced by mitochondrial malfunction and oxidative stress, and they also suggested that antioxidant medications are probably conducive to preventing and curing pain [29]. For example, activation of Nrf2 mediates the antiallodynic effect of EA on the rat model of type I complex regional pain syndrome by reducing local oxidative stress and inflammation [30]. In this study, it was uncovered that EA alone or EA plus miR-331-5p antagomir significantly increased the pain threshold of animals treated with rotenone, and also interestingly prolonged tail flick latency by contrast to the control group.

Rotenone represses mitochondrial ETC complex I to impair adenosine triphosphate production while strengthening reactive oxygen species (ROS) generation [31]. The process where ROS induces oxidation of polyunsaturated fatty acids is defined as lipid peroxidation [32]. Lipid peroxidation produces MDA, a genotoxic byproduct capable of creating stable microtubules through the coupling with DNA bases plus proteins, which can cause neuroinflammation and cell death. The results of the biochemical tests in this study showed that rotenone raised the MDA level in the hippocampus. Furthermore, rotenone dramatically reduced the activities of GPx and SOD in the rat hippocampal regions. The primary antioxidant enzymes that are crucial in scavenging and getting rid of free radicals are SOD and GPx [33]. Since rotenone inhibits mitochondrial complex I and destroys the antioxidant system, it can therefore cause an accumulation of free radicals and oxidative damage. EA alone or EA plus miR-331-5p antagomir significantly decreased the MDA level while enhancing the hippocampal SOD and GPx activities in rats, consistent with the findings of Li et al.[20] that EA reduced the MDA level in PD models. By the deactivation of free radicals and ROS as well as the boosting of endogenous antioxidant enzymes, EA may lessen lipid peroxidation.

It is well known that rotenone induces motor disorders by selectively degenerating dopaminergic neurons from the substantia nigra pars compacta of the PD model. The PD-associated cognitive impairment often has a correlation with hippocampal neuronal injury [34]. According to recent studies, rotenone also causes hippocampal neuronal apoptosis in the PD model in addition to memory impairment [35]. It was found by histological analysis through the present research that in the hippocampal CA1, CA3, and DG regions, rotenone exacerbated neuronal loss. By triggering microglia, rotenone contributes to the neuroinflammatory response and disrupts the BBB, which may result in neuronal death and memory loss [24]. In addition, EA alone or EA plus miR-331-5p antagomir mitigated the apoptosis of neurons from the CA1, CA3 and DG regions of hippocampus.

Concerning the cognitive function, BDNF functions in neuronal survival, neurogenesis and synaptic plasticity as an essential neurotrophin [36]. Cognitive impairment may be the result of neuronal death caused by decreased levels of BDNF [37]. In this study, the hippocampal BDNF expression decreased in rats administered with rotenone, consistent with previous findings, revealing that rotenone can lower the hippocampal BDNF expression [38]. Nevertheless, the exact chemical mechanism behind the reduction in BDNF expression after rotenone therapy remains unclear. Moreover, it was uncovered by the present research that EA could ameliorate the rotenone-induced reduction in hippocampal BDNF expression. This signifies that EA achieves a neuroprotective effect possibly by increasing the hippocampal BDNF expression.

Nevertheless, this study is limited. Whether there is a synergistic relationship between EA and miR-331-5p antagomir is not studied, which still needs further validation.

In conclusion, EA had a positive impact on non-motor deficits resulting from rotenone-induced neurotoxicity. EA reduced rotenone-induced thermal hyperalgesia and memory impairment. Furthermore, EA reduced the hippocampal MDA (lipid peroxidation product) content and prevented neuronal injury in rats treated with rotenone. These findings demonstrate that the antioxidant properties of EA partly mediate its ability to ameliorate memory impairment and neuronal injury induced by rotenone.

Declarations

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

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Competing Interests: There is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The article, tables and figure were not written/created by AI and AI-assisted technologies.

Authors' Contributions: Y. Y. designed and supervised the study,

and significantly revised the paper. D. L. performed this study and drafted the paper. Both authors have approved the submission and publication of the paper.

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Research Article

Effect of Nettle Extract on Metabolic Processes in Piglets During Weaning

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Abstract

The article deals with the use of a plant extract to improve metabolic processes and the adaptive capacity of piglets during the weaning period. The study focused on the effects of a 40% ethanolic extract of nettle *(Urtica dioica* L.) on hematological and biochemical parameters in the blood of piglets. The piglets were divided into two groups - control and experimental with 9 animals in each group. From 14 days of age until weaning, the experimental animals were fed with the nettle extract at a rate of 6 mg/kg body weight for 22 days. Blood samples were collected at 14, 36, and 42 days of piglets' age. The study found that the nettle extract improved the blood respiratory function and positively influenced protein and energy metabolism in piglets. The nettle extract led to increased hemoglobin concentration, erythrocytes and leukocyte count, total protein, transaminases activity, glucose concentration, alkaline phosphatase and creatine kinase activity. The piglets fed with nettle extract showed a 10% higher live weight and average daily gain at 42 days of age, and a 12% higher safety rate compared to piglets fed with a standard diet. The results indicate that adding 40% nettle extract to the standard piglets' diet can stimulate metabolic and adaptive processes, as well as improve productive performance during the critical period after weaning.

Keywords: *Urtica dioica* L. extract, biochemical and hematological parameters, weaning stress, piglets

Introduction

Numerous studies have shown that weaning is the most challenging period for pigs, affecting their welfare and productivity significantly [1-3]. During this time, piglets are exposed to several factors, including psychosocial (separation from the mother and movement to new groups), immunological, infectious, metabolic, and nutritional stress. These factors often result in diarrhea and intestinal damage, which can negatively impact the survival rate of young animals. The post-weaning mortality rate can range from 6-10% and can sometimes go up to 20%. To address this, animal nutritionists have been working to optimize feed composition to meet the needs of newly weaned pigs and have been exploring various nutritional factors and housing conditions to promote piglet health [4-6].

Herbs and essential oils are used in pig nutrition due to their antibacterial, anti-inflammatory, antioxidant, and antiparasitic properties. The use of herbs in pig nutrition has shown many positive results, such as improving the taste of feed, increasing appetite, regulating digestion and metabolic processes, and lengthening intestinal villi, which improves the digestibility and digestion of the feed ration [7,8]. Researchers have also found that herbs decrease post-weaning diarrhea in piglets by reducing the number of Escherichia coli and ammonia emissions [9].

Common nettle *(Urtica dioica L.)* is a wild herbaceous perennial flowering plant that grows in Europe, Asia, North Africa, and North America. Nettle is known for its anti-inflammatory, anti-proliferative, antioxidant, analgesic, anti-infective, hypotensive, and anti-ulcer properties. It can reduce the intensity of hormonal changes in the body, act as a coagulant, and be used to make fertilizers and insecticides. The plant's multifaceted chemical composition, which includes carboxylic acids, amino acids, lipids and fatty acids, nitrogen-containing compounds, essential oils, steroids, lectins, lignans, coumarins, histamine, pigments, vitamins, tannins, flavonoids, trace elements,

and macronutrients, determines its high pharmacological properties [10,11]. Because of the vast array of natural biologically active compounds that make up nettle, and the large amounts of environmentally friendly raw materials available on almost all continents, it's possible to isolate these compounds and develop new drugs and food additives from nettle extract. These extracts can increase adaptive capacity, and correct metabolic disorders in animals and humans, making this research extremely relevant [12,13].

The study aimed to determine the effect of ethanol extract of common nettle on some biochemical, hematological, and productive parameters in piglets during weaning.

Material and Methods

Ethical Statement

The permission to perform the experiments was obtained from the Bioethics Committee of the Institute of Animal Biology NAAS of Lviv, Ukraine, approval No 77 of 20 December, 2021. All experiments were conducted according to the EU Directive 2010/63/EU for animal experiments.

Experimental Plant, Collection, Identification and Preparation

The common nettle *(Urtica dioica L.)* was harvested in the Skole district, located in the Carpathian highlands. The plant was identified as the common nettle *(Urtica dioica* L.) using the atlas-identifier of plants of Ukraine [14]. Nettle air parts were dried under normal conditions (in a dark place, temperature 20-25°C, relative humidity 30-60%). The dry material was then crushed to a particle size of 1.5 mm and placed in the extractor. The extraction process took eight days at a temperature of 20°C. We used a classical maceration with the ratio of raw material to 40% water-alcohol solution as an extractant (1:20, m/v). After extraction, the nettle extract was filtered and dehydrated using a rotary evaporator until it acquired a powdery form. For the experiment, the dry extract was used.

Experimental Animals and Design

The experiment was performed on 14-day-old piglets of a large white breed with a live weight from 5.18 to 5.48 kg. After farrowing two groups (control and experimental) were formed: 3 sows with piglets in each group. Each sow was kept in a separate cage with piglets (8-10 heads). On the 35th day after birth, piglets were weaned and each litter was kept in a separate weaner cage. Animals were fed a standard diet *ad libitum*, using a premix from Sano (Ferkengold Forte) for weaned piglets with free access to feed and water.

Piglets of the experimental group (Nettle) received a 40% extract of nettle in the dose of 6 mg/kg of body weight. The feed was mixed with a dry nettle extract and added to the feeders each morning for a group of animals in the cage from the 14th day after birth and to the weaning day (35th day of age). The extract feeding period lasted 22 days. At the same time, sows did not have access to the feed crib. The piglets of the control group (Control) were kept on a standard diet. The experiment lasted 30 days. Blood of piglets for the study (3 heads from each cage, 9 animals from the group) was taken before morning feeding from the anterior vena cava at 14, 36 (1 day after weaning), and 42 days of age (7 days after weaning). The survival and morbidity of piglets of both groups were monitored during the experiment. At the beginning and at the end of the experiment, the piglets were weighted.

Sampling Procedures

Piglets' blood and plasma were studied. 1% solution of heparin was used as an anticoagulant. The blood plasma was separated by centrifugation at 700 g for 15 min. Hematological parameters (number of erythrocytes and leukocytes in the Goriayev chamber) were determined in the blood and hemoglobin concentration was studied by hemoglobin-cyanide method [15].

Biochemical Analysis

The total protein in blood plasma was determined by the Lowry method [16], glucose concentration was measured by the glucose oxidase method [15], the activities of the creatine kinase (CK, EC 2.7.3.2), alkaline phosphatase (ALP, EC 1.11.1.7), alanine aminotransferase (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) - using the kits "Simko LTD". The absorbance values were measured on a spectrophotometer "Unico" 1205 (USA).

Statistical Analysis

The result among the groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. Statistical difference was considered significant at $P<0.05$. Results were expressed as mean ± Standard error of mean.

Results

In the animals of the experimental group, nettle extract caused a significant increase in hemoglobin concentration by 6% ($P<0.001$) and 12% ($P<0.001$) on days 1 and 7 after weaning from sows (*Fig. 1*).

During the experiment, it was observed that 36 and 42-day-old piglets in the experimental groups had 8% ($P<0.01$) and 7% ($P<0.01$) more erythrocytes than the control group, respectively. In addition, the number of leukocytes in the experimental group on the 1 day after weaning was found to be 5% higher than that of the control group ($P<0.001$) *(Fig. 2-A,B)*. At 42 days of age, both the

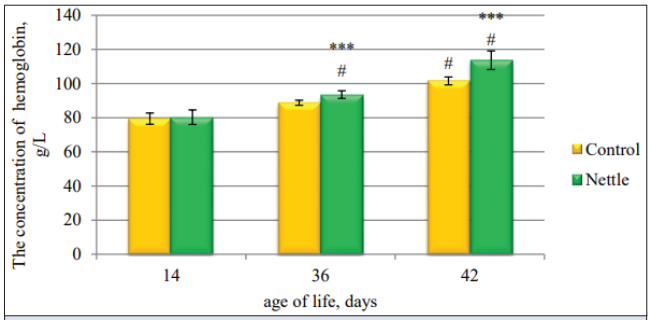

Fig 1. The concentration of hemoglobin in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** P<0.001); # the differences are significant compared to 14-day-old animals (# P<0.05; ## P<0.01; ### P<0.001); Control - control group of piglets; Nettle - experimental piglets, which were fed additionally to the standard diet 40% extract of nettle *(Urtica dioica* L.) in the dose of 6 mg/kg of body weight

control and experimental groups showed a significant increase in the concentration of Hb (P<0.05) *(Fig. 1)* and the number of erythrocytes (P<0.01) *(Fig. 2-A)*. Similarly, the number of leukocytes increased significantly on days 36 and 42 of life in both groups compared to the beginning

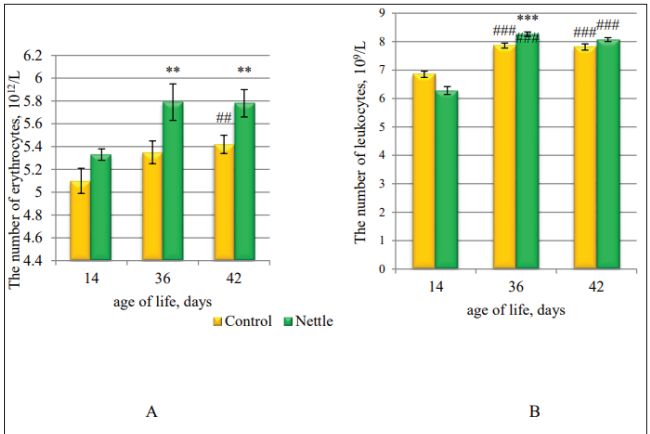

Fig 2. The number of erythrocytes (A) and of leukocytes (B) in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** - P<0.001); # - the differences are significant compared to 14-day-old animals (# P<0.05; ## P<0.01; ### P<0.001)

of the experiment, with an average increase of 1.3 times (P<0.001) *(Fig. 2-B)*.

Our results have shown that at 36 and 42 days of age, the experimental group of piglets showed a significant increase in the content of total protein in their blood plasma by 15% (P<0.001) and 6% (P<0.001) respectively, compared to the control. It's worth noting that the total protein in the control group of piglets significantly decreased by 5% (P<0.01) on the first day after weaning, while the experimental piglets' concentration increased by 11% compared to the beginning of the study (P<0.001). By day 42, the total protein in the blood of animals significantly increased by 15% (P<0.001) in the control group and 23%

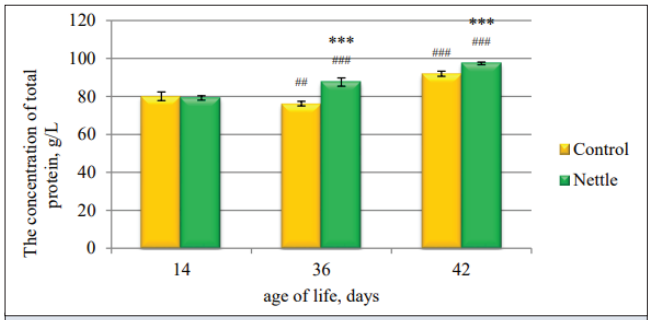

Fig 3. The concentration of total protein in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** P <0.001); # the differences are significant compared to 14-day-old animals (# P<0.05; ## P <0.01; ### P <0.001)

(P<0.001) in the experimental group compared to day 14 of life *(Fig. 3)*.

In the experimental group of animals, an increase in the activity of AST by 25% (P<0.001) and 15% (P<0.001) and ALT by 2.1 (P<0.001) and 1.5 (P<0.01) times was observed within the normal physiological range, on the 1 and 7 days after weaning, respectively, as compared to the control group. Both enzymes showed an increase in their activity at the same age periods in the blood of experimental piglets by 2 (P<0.001) and 1.5 (P<0.001) times, compared to 14-day-old animals However, in control animals, the activity of these enzymes remained at the same level throughout the experimental period *(Fig. 4)*.

At 1 and 14 days after weaning, a significant increase of glucose in the blood plasma of the experimental animals compared to the control by 11% (P<0.01) and 10% (P<0.01), respectively, was found. Moreover, the decrease in the content of this metabolite was found in 36-day-old control and experimental piglets by 21% (P<0.05) and 12 % (P<0.05), respectively, compared to the beginning of the study, as shown in *Fig. 5*.

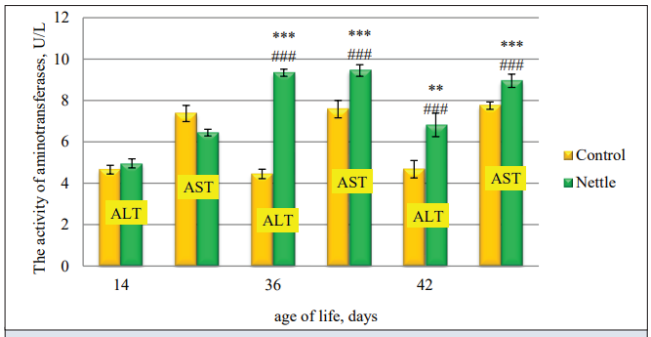

Fig 4. The activity of aminotransferases (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** P<0.001); # the differences are significant compared to 14-day-old animals (# P<0.05; ## P<0.01; ### P<0.001)

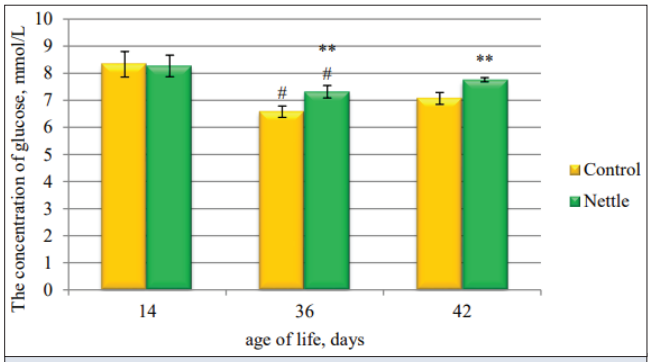

Fig 5. The concentration of glucose in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** P<0.001); # the differences are significant compared to 14-day-old animals (# P<0.05; ## P<0.01; ### P<0.001)

In the study, it was found that the activity of ALP and CK increased significantly in the blood of piglets subjected to nettle treatment at 36 and 42 days of age compared to animals on a standard diet. The activity of ALP increased by 25% (P<0.001) on the first day after weaning and by 10% (P<0.01) on the seventh day in comparison with control group, while decreasing in both groups of piglets by three times compared to 14-day-old animals (P<0.001) *(Fig. 6-A)*. The activity of CK was also found to be significantly increased in 36-day-old experimental animals by 1.4 times (P<0.001) and in 42-day-old animals by 1.8 times (P<0.001) compared to the control group. Furthermore, the activity of CK increased significantly in the blood of both control and experimental piglets by 1.4 times (P<0.001) and 2 times (P<0.001) respectively on day 1 after weaning. However, on day 14 in control piglets, the activity of CK decreased by 1.4 times (P<0.001), whereas in experimental animals, it remained 1.3 times higher (P<0.001) *(Fig. 6-B)*.

The performance of piglets of both groups was determined for the experiment. At 14 days after birth, both groups had a live weight between 5.18-5.48 kg. On day 7 after

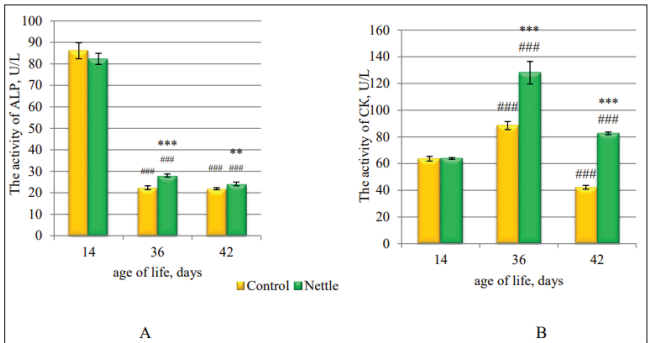

Fig 6. The activity of alkaline phosphatase (ALP (A)) and creatine kinase (CK (B)) in the blood of piglets (M ± SE, n = 9). * the differences are significant between the control and experimental groups of piglets (* P<0.05; ** P<0.01; *** P<0.001); # the differences are significant compared to 14-day-old animals (# P<0.05; ## P<0.01; ### P<0.001)

weaning (42 days of age), piglets in the control group had an average weight of 9.3 kg (with an average daily gain of 221 g), while piglets treated with nettle extract had an average weight of 10.25 kg (with an average daily gain of 244 g). Therefore, the addition of nettle extract to the piglets' diet led to a 10% increase in live weight and average daily weight gain compared to the standard diet. Moreover, the experimental group of piglets had a 12% higher survival rate than the control group.

Discussion

As already mentioned, weaning piglets from sows causes the simultaneous appearance of many stressors, which leads to low feed intake, intestinal disorders, inflammation, increased incidence of disease, and mortality, which causes significant economic losses for producers [1,17]. Researchers have pointed to a specific link between mitochondrial function, oxidative stress, and weaning stress, which causes a decrease in the activity of mitochondrial respiratory complexes in intestinal and hepatic tissues during the first week after weaning [3,18]. In our study, a decrease in the concentration of total protein was found in the blood plasma of piglets of the control group on the first day after weaning. One of the reasons for this decrease in protein may be damage to protein molecules as a result of oxidative stress. Hao Y et al.[19] found that protein hydroxyl, a marker of protein oxidative damage, significantly increased in the body of piglets on the 1 day after weaning.

In another part of the same study [20], we observed that weaning piglets from sows causes oxidative stress, resulting in an increase in the concentration of metabolites associated with free radical damage. Specifically, we revealed an increase in carbonyl groups of proteins on the first day and primary metabolites of lipid peroxides on the seventh day after weaning. Our findings indicate that the activation of free radical processes in piglets occurs due to an immature antioxidant system, leading to reduced activity of the enzymatic components - superoxide dismutase, glutathione peroxidase, and catalase - in their blood, as well as a decrease in the concentration of the non-enzymatic antioxidant - reduced glutathione.

Therefore, to stimulate the body's defenses and metabolic processes in one of the most critical moments of piglets' life, we used the nettle extract. It was found that the studied extract has a positive effect on the metabolic systems of animals. The increase in morphological parameters on days 1 and 7 after weaning compared to the control indicates the hematopoietic effect of nettle, rich in trace elements, especially Iron, Zinc, and Copper, on erythropoiesis (increase in the number of red blood cells within the physiological norm), antianemia effect (increase in hemoglobin concentration) and stimulation

of respiratory function in experimental animals compared to control ones. Some authors explain the positive impact of nettle on hematopoiesis by stimulating the absorption of minerals from the feed [21-24].

The positive effect of 40% nettle extract on the hematological parameters of piglets can be explained by the action of its components. These components include ascorbic acid, vitamin E (as a part of red blood cell membranes), B vitamins, organic acids (citric, malic, succinic, etc.), and Se. These components directly or indirectly affect the oxygen transport function of hemoglobin [13,25,26]. The protein-iron complex, which is formed with a sufficient amount of Fe, helps improve absorption through the small intestinal mucosa. This is extremely important for piglets during the weaning period. It's worth noting that the increase in the number of leukocytes in the blood of piglets after weaning within the normal range under the influence of nettle may indicate its immunomodulatory properties. This is due to the presence of compounds such as quercetin, kaempferol, and isorhamnetin in the nettle extract. Some authors noted [19,27,17] that these compounds promote the rapid proliferation of bone marrow cells and the release of young blood cells into the bloodstream.

The increase in the concentration of total protein in blood of experimental piglets especially on the 1 day after weaning, confirms the activation of its synthesis under the nettle action. The high content of organic acids, such as formic, citric, and ascorbic acids, provides a positive effect of the extract on redox and anabolic processes in the body of young animals. These data are consistent with the work of A. Szewczyk in 2006, where it was found that nettle extract used as a dietary supplement for finishing pigs affects protein synthesis and fat metabolism. It also changes the profile of fatty acids and lipids in the blood, reducing total cholesterol and triglyceride concentration [28].

The positive effect of nettle extract on protein synthesis is closely related to the processes of transamination. The increase in AST and ALT activity in our study indicates the stimulation of thermogenesis and gluconeogenesis in the experimental piglets' bodies. The intensification of these processes is confirmed by an increase in the glucose concentration in the blood of piglets. This is evidence of the hydrocarbon metabolism activation under the influence of nettle extract that is necessary for the organism of young animals during weaning [2,7].

The activation of energy and redox processes under the influence of nettle can be explained by the presence of citric acid (improving the absorption of Phosphorus by the body) and silicic acid and its salts (stimulating the absorption of Calcium). A large amount of anti-oxidant compounds (ascorbic acid, carotenoids and flavonoids) in nettle also contributes to the activation of these processes [12,29].

In a previous study, we analyzed the composition of 40% of the nettle extract that was added to the diet of the experimental piglets. The concentrations of biologically active substances found were as follows: polyphenols - 11924.38 mg/100 g, carotenoids - 0.127 mg/g, chlorophyll a - 0.513 mg/g, and chlorophyll b - 0.174 mg/g.

The high activity of ALP in the blood of young animals is explained by the intensive functioning of osteoblasts in bone tissue, which is due to the processes of active growth of the organism, especially in piglets of the experimental group. During this period, the activity of the enzyme in the blood increases due to bone isoenzyme. The higher activity of ALP in the blood of experimental animals is also explained by the increased phosphorylation processes due to better intake and absorption of phosphorus and calcium (increase in the free phosphate fund by increasing ALP activity). To stimulate redox and energy processes, a high level of ATP is also required. The ATP level is provided by an increase in the activity of the CK under the nettle extract action [4,30].

The activation of anabolic processes in the organism of piglets fed with 40% nettle extract explains the increase in their live weight and average daily gain. This is also a confirmation of the positive effect of nettle extract on the general physiological state of experimental animals, compared to piglets kept on a standard diet.

The data on the elevation of piglets' weight and safety under the influence of nettle extract are consistent with the results of other authors who have observed the improvement of productivity indicators. These results indicate the increased viability, activation of metabolic and immune processes in the body, better absorption of feed and its components due to a positive effect on the gastrointestinal tract and intestinal microflora, as well as suppression of the expression of proinflammatory cytokines [1,9].

The literature analysis revealed that most studies focused on adding herbal preparations, green nettle, or a combination of other herbs, to piglet diets. Less commonly, studies used aqueous nettle extracts. The effects of nettle extracts on the body can be explained by their impact on intestinal microflora. Therefore, we focused on the anti-stress and adaptogenic effects of nettle extract on various metabolic processes. To extract the optimal amount of antioxidants to enhance the resistance of young animals under stress, we utilized a water-ethanol mixture. Our research on nettle extracts, including aqueous and ethanolic (20%, 40%, 60%, 70%, and 90%), demonstrated that the 40% extract revealed the highest radical scavenging activity. Notably, there

is limited literature data on the effect of such an extract on farm animals during weaning stress. Considering the promising outcomes, further investigation of the impact of this specific nettle extract on animals under various stressful conditions is recommended.

In conclusion, it should be noted that the addition of herbal preparations, as well as any other biologically active compounds into the diet of farm animals of different productivity and age, should be considered comprehensively and follow one rule. According to this rule the positive effect of these additives on metabolism, general resistance and safety of the organism can occur only under optimal conditions of keeping, complete and balanced feeding and ensuring a high level of their welfare.

Thus, our study has shown that a powerful complex of natural biologically active substances contained in 40% ethanolic nettle extract causes activation of metabolic processes, improved digestibility of dietary nutrients and safety of piglets during weaning. Therefore, it is suggested to add nettle extract to the standard diet of young animals to stimulate metabolic and adaptation processes, as well as improve performance indicators of their body during critical periods of ontogenesis.

Declarations

Availability of Data and Materials: The data sets analyzed during the current study are available from the corresponding author (V. Havryliak) on reasonable request.

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Ethical Statement: The permission to perform the experiments was obtained from the Bioethics Committee of the Institute of Animal Biology NAAS of Lviv, Ukraine, approval No 77 of 20 December, 2021.

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Authors' Contributions: O.B. designed the research and collect the samples. V.H., O.Y. performed the experimental duties of this study and analyzed the data. O.B. and V.H. wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Genomic Diversity and Autozygosity-Based Signatures of Selection in Kangal Akkaraman Sheep via Genotyping-by-Sequencing

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Abstract

In this study, genome-wide variability and selection signatures in Kangal Akkaraman sheep were assessed by 238.103 bi-allelic single nucleotide polymorphisms (SNPs) recovered from genotyping-by-sequencing (GBS) libraries processed in Illumina HiSeq X Ten instrument. Summary statistics of genetic diversity such as minor allele frequency (MAF), observed (H_O) and expected (H_E) heterozygosity were estimated at 0.32, 0.29, and 0.30, respectively. A declining trend in effective population size was observed through generations in which the current population was estimated at 978 individuals 150 generations ago. 608 of 647 runs of homozygosity (ROH) islands were classified into ≤2 Mb. Strong selection signals were identified in thirteen genomic intervals overlapped with 17 protein-coding genes. The sheep quantitative trait locus (QTL) database confirmed that these genomic regions were associated with economically important traits such as milk content *(KCNH5, KCNH7, LRP1B, SNAPC1,* and *SYT16)* and fleece yield *(CCDC85A*, *EFEMP1*, and *PPP4R3B),* parasite resistance *(MMS22L* and *KLHL32),* fat deposition in the tail *(JAZF1, TAXIBP1, EVX1,* and *HOXA13),* and water-holding capacity *(KLHL1* and *DACH1).* This study implies that the Kangal Akkaraman sheep will play a vital role in developing some genotypes tolerant to environmental challenges, parasite infections, fat deposition, and water-holding capacity in the future. Still, the other native sheep should be screened to identify genomic regions under selection practices using high-density genetic data obtained from next-generation sequencing (NGS) platforms.

Keywords: Genetic variation, Genotyping-by-sequencing, Kangal Akkaraman, Molecular genotyping, Selection signals, Selective breeding

Introduction

Mainly distributed in Central Anatolia and nearby places, Akkaraman is the most raised breed with an approximately 40-45% proportion among the native Turkish small ruminant population [1]. Both systematic and non-systematic selection applications have been done in the Akkaraman breed by breeders in which Kangal Akkaraman has been derived from systematic selection management [2]. Kangal Akkaraman breed has been developed to increase body weight phenotype [3], whilst it is raised for meat and milk production in Sivas and nearby provinces [4]. There are ongoing debates about whether Akkaraman-derived sheep are distinct enough from their ancestral population in terms of morphology and genetic structure to categorize them as "variety" or "breed". For example, a documentary published by the General Directorate of Agricultural Research and Politics (GDARP) has categorized Kangal Akkaraman as a distinct breed [4], whereas several studies have stated that this population remains a variety of Akkaraman [2,3]. It seems that further studies using both detailed phenotypic records and whole genome-based phylogenetic analyses are required to enlighten this obscurity.

Variations in the genome are the main reason causing differences among individuals in terms of morphological, psychological, behavioral, and adaptive traits. These variations have been utilized by human beings for diverse purposes throughout history. As highlighted by the Food and Agriculture Organization of the United Nations (FAO) [5], thanks to the manipulation of genetic variations for environmental requirements and economic interests, approximately 8.774 breeds belonging to 38 major animal species have been developed since domestication. Variations in a population including all breeds and varieties are called genetic diversity. Farmers exert great efforts to keep genetic diversity at an optimal level to maintain

their production for current and future demands. In fact, genetic variations in related genes not only improve survival traits in livestock species but also play a pivotal role in selection studies to enhance economically important traits such as meat and milk. On the contrary, intensive selection practices conducted for a specific purpose across several generations may lead to pressure over the genome. This kind of selection is of potential to significantly decrease genetic variations, known as selection signatures, not only in the corresponding genes but also in nearby neutral genomic regions [6].

Periodically monitoring genetic diversity is a beneficial way to shape conservation studies as well as selection practices against environmental factors negatively affecting sustainable animal production in the future. Depending on the type of genetic data, genetic diversity could be estimated via numerous statistics such as the number of alleles, number of effective alleles, nucleotide diversity, minor allele frequency (MAF), observed heterozygosity *(H_O),* expected heterozygosity *(H_E),* and inbreeding level [7]. On the other hand, selection signatures studies are promising to obtain a deeper knowledge of the past breeding history of farm animals and detect regions subjected to recent and long-term selection pressure. Several alternative approaches are available to scan selection signatures at the genome-wide level within and between populations in which runs of homozygosity (ROH) is one of the popular approaches. ROH is defined as the DNA segments holding consecutive homozygous genotypes in an individual due to parents transmitting identical haplotypes to their offspring [8]. Longer ROH segments indicate recent inbreeding, whereas shorter consecutive homozygous segments are considered a sign of long-term selection pressure [9]. However, both analyses (genetic variability and selection signatures) require high-density genomic data to effectively carry out selection and conservation studies.

Fortunately, thanks to rapid advances in molecular genetics, single nucleotide polymorphism (SNP) arrays and next-generation sequencing (NGS) platforms have been developed to recover genetic data across the whole genome. SNP arrays are commonly used for genotyping farm animals due to their simplicity, whereas they possess some disadvantages. Indeed, as highlighted by Bilginer et al.[7], SNP arrays have been developed based on some reference breeds and numerous variations related to environmental adaptation in local breeds could be neglected. Unlike, NGS platforms may overcome this bias since genetic data are recovered randomly across the whole genome. Of these platforms, genotyping-by-sequencing (GBS), one of the reduced representations of genomic libraries, relies on a single restriction enzyme following the barcode ligation and pooling processes [10]. Short reads including barcode information could be processed via different sequencing platforms with a single-end or paired-end mode. Restriction enzyme-based techniques including GBS not only allow for facilitating the complexity of the whole genome but also create an opportunity for higher sample multiplexing which significantly decreases sequencing costs [11].

Although NGS platforms are becoming cost-efficient and more applicable, there is a lack of studies in the literature to focus on screening genetic diversity and selection signatures in Kangal Akkaraman sheep. In this context, this study aims to reveal genetic diversity, ROH characterization, and selection signatures in Kangal sheep via high-density SNP data obtained from GBS library preparation combined with the Illumina HiSeq X Ten sequencing platform.

Material and Methods

Ethical Statement

This study was approved by the Akdeniz University Animal Experiments Local Ethics Committee (Approval no: 1691/2024.04.004).

Sampling and DNA Extraction

Twenty-five animals (5 male and 20 female) belonging to the Kangal Akkaraman breed were sampled from four different herds reared in Sivas province. Oral interview with breeders was utilized to select unrelated animals. Blood samples taken from the jugular vein were subjected to the GeneJET Genomic DNA Purification Kit (Thermo K0721) following the manufacturer's recommendations in order to isolate DNA. DNA quality and quantity were checked by NanoDrop 2000 (Thermo Scientific) and Qubit 4™ (ThermoFischer Scientific), respectively, in which DNA quality ranged from 1.79 to 1.83 at 260/280 OD, while DNA concentrations varied between 34.7 ng/µL and 38.2 ng/µL across the samples. Isolated DNA was optimized for all samples at 30 ng/µL before genomic library preparation was performed.

GBS Library Preparation and Illumina Sequencing

GBS libraries were prepared by using the Eco*RI* (New England Biolabs) restriction enzyme and 25 universal indexed adapters recommended by the Illumina. Briefly, DNA was digested overnight with restriction enzyme at 37°C. Adapters were ligated to DNA fragments using T4 DNA ligase (Invitrogen) at 22°C for an hour and cleaned with AmPureXP (Beckman Coulter) beads. DNA libraries were enriched with the Polymerase Chain Reaction (PCR) technique and pooled libraries were sequenced via single-end mode in the Illumina HiSeq X Ten platform (1x150 base pair).

Variant Calling and Filtering

The Stacks 2 program [12] was employed to assign short reads to individuals according to their barcode information. Assigned reads were processed by the fastp software [13] with default parameters for quality trimming and adapter removal. Burrows-Wheeler Aligner [14] was run with default settings to align clean reads to the reference genome of *Ovis aries* (ARS-UI_Ramb_v3.0). BCFtools pipeline [15] was chosen to call the variants in which only bi-allelic SNPs passing the criteria of read depth ($20 \leq D \leq 500$) and base quality score ($Q \geq 20$) were kept. On the contrary, all the InDels and SNPs not located on autosomal chromosomes were excluded from the data set. The remaining SNPs were processed via PLINK 1.9 software [16] in order to recover SNPs with high genotyping rates (--geno 0.1) and MAF values (--maf 0.05). In the last step, animals with a low genotyping rate (--mind 0.1) were excluded to obtain the final data set.

Statistical Analysis

Genetic diversity parameters such as MAF, H_O, and H_E, in the Kangal Akkaraman breed were calculated in PLINK 1.9 software [16]. The historical effective population size was estimated via SNeP v.1.1 tools with default parameters described by Barbato et al.[17]. The results of effective population till 150 generations ago were visualized by the *plot* function implemented in the R environment [18]. The detectRUNS package [19] implemented in the R environment [16] was run with a consecutive runs approach to analyze genomic inbreeding value derived from ROH (F_{ROH}), ROH characterization, and selection signatures. ROH islands were defined according to the following criteria: i) the minimum number of consecutive SNPs was optimized at 15, ii) the minimum length of a ROH was set to 1 Mb, iii) the maximum gap between consecutive homozygous SNPs was 1 Mb, iv) the maximum two SNPs with missing genotypes and up to one heterozygous were allowed in a ROH. Based on their physical length, each ROH island was categorized into 0 to <2 Mb, 2 to <4 Mb, 4 to <8 Mb, 8 to <16 Mb, and ≥16 Mb clusters. The number of ROH island per each aforementioned ROH length class and chromosomes were calculated. SNPs passing ROH characterization were visualized for all autosomes in the Manhattan plot command of the "*qqman*" package [20] implemented in the R environment [18]. 0.1% of SNPs based on empirical distribution were considered to be under selection pressure Genomic windows of 200 kb (100 kb upstream and 100 kb downstream of the significant SNPs) were screened to detect overlapping protein-coding gene segments. The genes overlapping these segments were confirmed via the *Genome data viewer* module of the *National Institutes of Health (NCBI)* platform [21] by choosing the options of ARS-UI_Ramb_v3.0 assembly. To validate the effects of genes under selection pressure on phenotypic traits, the sheep QTL database *(SheepQTLdb)* [22] was checked.

Results

In this study, nearly 185 million cleaned reads ranging from 5.7 to 12 million per individual were recovered by the GBS technique. A large part of clean reads (94%) were mapped to the reference genome successfully. A total of 1.337.343 SNPs and 86.656 InDels were obtained in the variant calling process, whereas only 238.103 bi-allelic SNPs and 22 individuals passed the filtering criteria. Genotyping rate was 100% indicating that 22 animals possessed alleles regarding 238.103 SNPs without missing genotypes.

Genome-wide analysis revealed that observed heterozygosity (0.29) was slightly lower than expected heterozygosity (0.30) in Kangal Akkaraman sheep. MAF value was estimated at 0.32, while the ROH-based inbreeding coefficient (F_{HOM}) was close to zero (0.01).

The historical changes in population size were assessed via linkage disequilibrium (LD) in which a declining trend was detected across generations. For example, the current 22 individuals turned out to be descended from 100 individuals 13 generations ago *(Fig. 1)*. Moreover, 54 generations ago, the ancestral population was estimated at 378 individuals, whereas the current population was represented by 978 animals 150 years ago *(Fig. 1)*.

Via consecutive runs algorithm, a total of 647 ROH islands were detected. According to their physical length, 608 and 39 were clustered into 0-2 and 2-4 Mb classes, respectively. No ROH islands were detected to be higher than 4 Mb in the Kangal Akkaraman breed. At the chromosome level, the lowest (2) and highest (97) numbers of ROH islands were detected in autosome 19 and 12, respectively. Strong selection signals were detected at thirteen different genomic intervals distributed to ten chromosomes *(Fig. 2)*.

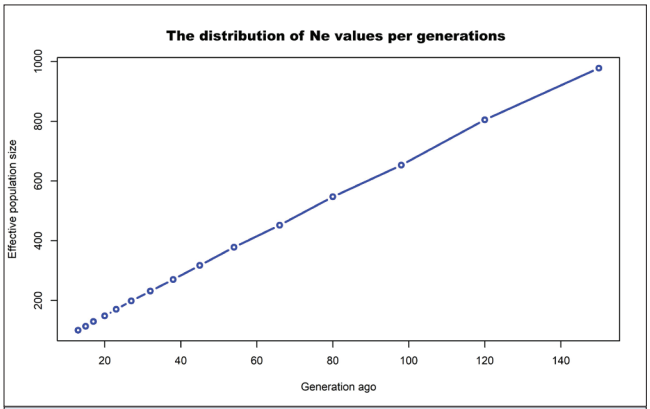

Fig 1. The estimated effective population size in the Kangal Akkaraman sheep for the past 150 generations

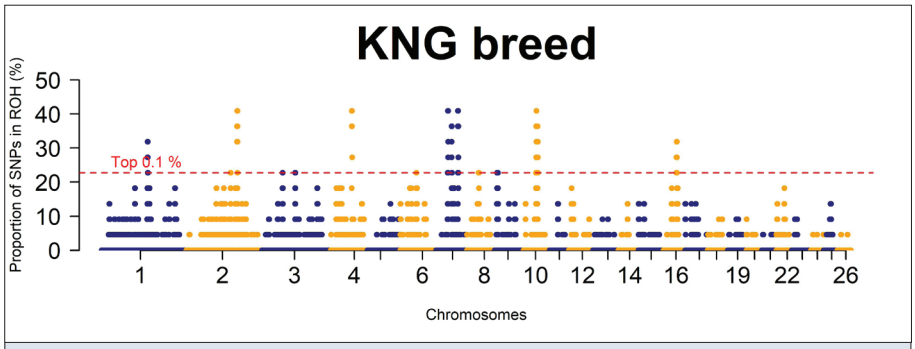

Fig 2. Manhattan plot of the distribution of ROH segments across the autosomal chromosome in Kangal Akkaraman sheep (red line indicates the threshold of top 0.1% SNPs in each breed)

Table 1. *ROH-based genes under selection pressure in Kangal Akkaraman sheep and their effects on phenotype*

Chr	SP	EP	NS	Corresponding Gene(s)	QTL-Related SNPs	Effect(s) on Phenotype
1	151258693	152646935	18	-	-	-
2	146570469	146940231	16	*KCNH7*	2	Milk content
	167861018	169409445	25	*LRP1B*	6	Milk content, fat density, and total protein level in blood
3	67843043	68475055	35	*CCDC85A*, *EFEMP1*, and *PPP4R3B*	5	Fleece yield and bone density
	109115550	110132697	37	-	-	-
4	69828415	71081065	28	*JAZF1*, *TAX1BP1*, *EVX1*, and *HOXA13*	6	Tail fat deposition
6	53545871	53772381	15	-	-	-
7	38305455	39658987	25	*MDGA2*	-	-
	71989011	73094768	23	*SNAPC1*, *SYT16*, and *KCNH5*	6	Milk content, bone density, and body circumference
8	38930308	39921843	15	*MMS22L* and *KLHL32*	2	Fecal egg count
9	10156994	11032974	34	-	-	-
10	41451793	47763646	63	*KLHL1* and *DACH1*	3	Water holding capacity
16	43064491	44451481	29	-	-	-
Total	-	-	363	17	30	-

Chr: *Chromosome,* ***SP:*** *Start Position,* ***EP:*** *End Position,* ***NS;*** *Number of detected SNPs*

Based on the empirical distribution of the proportion of SNPs in ROH islands, a total of 363 SNPs passed the top 0.1% criterion *(Table 1)*. The lowest number of SNPs (15) under selection pressure was observed in chromosomes 6 and 8, while 63 SNPs were identified in chromosome 10. Although strong selection signals were detected in chromosomes 1, 6, and 9, no protein-coding genes were identified in the related genomic intervals *(Table 1)*. On the other hand, a total of seventeen protein-coding genes *(CCDC85A, DACH1, EFEMP1, EVX1, HOXA13, JAZF1, KCNH5, KCNH7, KLHL1, KLHL32, LRP1B, MDGA2, MMS22L, PPP4R3B, SNAPC1, SYT16,* and *TAX1BP1)* were present in the remaining genomic intervals *(Table 1)*. It has been confirmed via the sheep QTL database that a total of 30 QTL-associated SNPs overlapped with genomic regions under selection pressure which turned out to be associated with several phenotypes such as survival traits (water holding capacity and fat deposition in tail), resistance to parasites, morphology (bone density and body circumference), and economically important traits (milk content and fleece yield).

Discussion

The genetic diversity parameters in native Turkish sheep breeds have been mainly monitored via microsatellite markers [2,3], while two studies utilizing the bi-allelic SNP

data have been recently published to assess genome-wide genetic variability in several Anatolian sheep populations [23,24]. An Illumina OvineSNP50 array-based genotyping revealed that heterozygosity ranged from 0.34 to 0.35 with negative inbreeding values among three native sheep populations known as Sakız, Karakaş, and Norduz [23]. Another recent study conducted by Karsli [24] confirmed that the average MAF value was 0.31, whereas heterozygosity ranged from 0.29 to 0.31 in four Anatolian sheep populations (Akkaraman, Güney Karaman, Karakaş, and Morkaraman) which were genotyped via ddRADseq libraries sequenced with Illumina HiSeq X Ten instrument. Besides, negative inbreeding coefficient values were reported for all sheep populations [24]. The current study showed consistent results in terms of inbreeding value (0.01) with previous studies. It is not surprising to detect a low inbreeding in this study because unrelated animals were chosen based on oral interviews with farmers. A lower heterozygosity (0.29) was detected in the Kangal Akkaraman breed compared to findings reported by Bayraktar [23], while similar values of MAF and heterozygosity were observed with findings declared by Karsli [24]. This finding could be attributed to differences between genotyping tools in which SNP arrays scan the previously known variations while the methods of the reduced representation of the whole genome such as GBS and ddRADseq randomly detect genetic variations which could be less polymorphic across the studied populations. As highlighted by Bilginer et al.[7], who comprehensively reviewed several molecular genotyping methods for revealing genetic diversity, NGS platforms such as ddRADseq and GBS are advantageous over microsatellites and array technologies due to covering a larger part of the genome and allowing for variations specific to local populations. Indeed, Bayraktar [23] estimated genomic diversity via 46.314 SNPs in Sakız, Karakaş, and Norduz, while the current study (238.103 SNPs) and Karsli [24] (296.097 SNPs) benefit from higher-resolution genetic data to calculate genome-wide genetic variability in Anatolian sheep.

It was concluded from LD-based analysis that the effective population size of the Kangal Akkaraman breed has decreased from one generation to another. The current 22 individuals were validated to be represented by approximately 1000 animals 150 generations ago. Unfortunately, the historical effective population size of native Turkish sheep breeds has not been calculated via genetic data till now. However, several studies mentioned that the effective population size of native Anatolian sheep breeds has decreased due to uncontrolled breeding systems [2,25], while some breeds such as Güney Karaman and Çine Çaparı have been reported to be on the brink of extinction [26,27].

Compared to genetic diversity studies, revealing genomic regions under selection pressure is a new field of study in Türkiye. Indeed, Demir et al.[6] have recently identified several genes related to visual modality *(LGSN),* olfaction *(MOXD2, OR4C1F,* and *OR4C1E),* and immune response *(TRBV3-1* and *CLDN10)* were under selection pressure in six native Turkish cattle breeds which were genotyped with 211.119 SNPs recovered from ddRADseq technique. On the contrary, NGS-based studies aiming to assess selection signals in numerous livestock species such as sheep, goats, chickens, and geese reared in Türkiye are required to obtain deeper knowledge about their past breeding practices. Therefore, the current study is of significant potential to enlighten genomic regions under selection practices. Indeed, strong selection signals were detected in seventeen protein-coding genes in the Kangal Akkaraman breed which were further confirmed to possess a total of 30 QTL-associated SNPs. A survey of the sheep QTL database validated that some of these genes *(KCNH5, KCNH7, LRP1B, SNAPC1,* and *SYT16)* were associated with economically important traits such as milk content and fleece yield. On the other hand, it is known that native Turkish sheep breeds including Kangal Akkaraman are well-adapted to environmental challenges [28]. Indeed, this study revealed that several genes under selection pressure cover some fixed SNPs related to parasite resistance *(MMS22L* and *KLHL32),* fat deposition in the tail *(JAZF1, TAXIBP1, EVX1,* and *HOXA13),* and water-holding capacity *(KLHL1* and *DACH1).* Moreover, a large part of ROH islands (93.97%) were shorter than 4 Mb indicating that the corresponding genes have been subjected to selection practices for the long term which allowed animals to develop adaptation against environmental challenges. Of these environmental challenges, parasite infections negatively affect health, welfare, and productivity in susceptible animals [29], whereas tolerant animals are of the ability to maintain their production level regarding economically important yields. Fat deposition and water storage capacity play a vital role in surviving in animals reared in grassland. Indeed, as highlighted by Xu et al.[30], fat deposition is an indispensable element for animals thereby harsh environmental stressors such as drought seasons, extreme cold winters, and food shortages could be tolerated by conserving a valuable energy reserve. Due to ongoing global warming, on the other hand, water scarcity will be one of the most threatening environmental challenges in arid and semi-arid regions by causing negative effects on health and reproduction in sheep [31].

In conclusion, the Kangal Akkaraman breed was screened at a genome-wide level to investigate genomic diversity, effective population size, and selection signatures. It was observed that the Kangal Akkaraman breed conserves

sufficient genetic variability across the genome, while effective population size is declining through generations. The authorities should take solid action to prevent this trend in the future. Moreover, the Kangal Akkaraman breed should be subjected to comprehensive conservation programs due to their adaptability to harsh environmental conditions. Indeed, several studies have mentioned that native Turkish sheep populations are well-adapted to their environment. This study conducted at the genome-wide level confirms that several genes associated with environmental adaptation such as parasite resistance, fat deposition, and water storage capacity have become fixed in the Kangal Akkaraman breed. Since the negative impacts of global warming will become more threatening in the future, fat deposition and water storage capacity will be an indispensable part of selection studies. In this context, the Kangal Akkaraman breed will play a vital role in developing selection strategies against environmental challenges. It is noteworthy that the other native Turkish sheep breeds may hold some advantageous genotypes related to survival traits. Therefore, it is recommended that further studies should focus on unraveling fixed genomic regions in other native Anatolian sheep via high-density SNP data obtained from NGS platforms.

Declarations

Availability of Data and Materials: Genomic data used in this study are available from the corresponding author (E. Demir) upon a scientific request.

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RESEARCH ARTICLE

Phylotypes, Antibiotic Resistance and Biofilm Formation Determined in Enteropathogenic *Escherichia coli* Isolates in Diarrheic Cats

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Abstract

This study investigated the phylotypes, biofilm formation abilities, antibiotic resistance of enteropathogenic *Escherichia coli* (EPEC) isolates from cats with diarrhea. From 37 diarrheal cats, 28 *E. coli* isolates were obtained using conventional methods. Pathotypes and phylotypes were determined via PCR, biofilm formation potential via Congo Red Agar, and resistance profiles against eight antibiotics were examined using the disk diffusion method, evaluated according to the Clinical and Laboratory Standards Institute (CLSI). Chi-square tests assessed relationships between phylogenetic groups, biofilm formation, and MDR statuses. The results were considered statically significant at a 95% confidence interval and significance level of $P<0.05$. Pathotyping studies showed that 46% (13 isolates) of the isolates were EPEC. 93% of isolates were phylotyped. Seven phylotypes were detected: B2 (22%), C (18%), B1 (18%), D (14%), A (11%), E (7%) and F (3%). Of the isolates 39% formed biofilms and 86% were MDR. No significant association was found between pathotype and biofilm formation or MDR. However, a significant relationship was noted between pathotype EPEC and phylogenetic group B2. The correlation between EPEC pathotype and phylotype B2 in diarrheic cats suggests high pathogenic potential. Multidrug resistance, even in non-biofilm forming isolates, complicates treatment and poses public health risks, underscoring the need for detailed evaluation of *E. coli* diversity and zoonotic pathogens.

Keywords: Antibiotic resistance, Biofilm, Cats, Enteropathogenic *Escherichia coli*

INTRODUCTION

Escherichia coli, a prominent cause of diarrhea, is classified into various groups based on virulence factors, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) [1,2]. EPEC, initially associated with childhood diarrhea epidemics, remains a significant cause of morbidity and mortality among infants and young children [3,4]. The distinction between typical (tEPEC) and atypical (aEPEC) strains is based on the presence of the *E. coli* adherence factor (EAF) plasmid [1,5], encoding bundle-forming pilus (BFP) and plasmid-encoded regulator (PER). tEPEC strains exhibit *eae*+ *bfpA*+ *stx*- genotype and attaching and effacing (A/E) phenotype, whereas aEPEC strains lack *bfpA*, with a genetic basis of *eae*+ *bfpA*- *stx*- [5,6]. Despite the positive impact of cats on human well-being, the potential for zoonotic disease transmission underscores the need for vigilance [7]. Türkiye, with an estimated 4 million cats and 2 million dogs as pets, witnesses significant pet ownership [8]. Cats, constituting 60% of identified pets, are associated with high levels of antimicrobial resistance in *E. coli* isolates, reflecting the challenges posed by indiscriminate antibiotic usage [9]. Environmental factors, including the presence of small children in cat-owning households, further contribute to *E. coli* colonization [7,9,10]. Cats represent potential reservoirs of antimicrobial resistance, increasing the risk of *E. coli* transmission to humans through close contact. Recent classification of *E. coli* into eight phylotypes based on genetic markers provides insights into their genetic diversity and pathogenic potential [11]. Phylotypes B2 and D are associated with severe extraintestinal infections, while A, B1, C, and E are mostly commensal or apathogenic [11-13]. Biofilm formation, facilitated by bacterial adhesion, contributes to virulence and recurrent infections [14-16]. Congo Red Agar (CRA) and other methods quantify biofilm formation capacities [17,18]. The shedding of *E. coli* in cat feces poses

a significant zoonotic risk, underscoring the importance of understanding EPEC prevalence and characteristics [19]. Given geographic variability, investigating cat-derived EPEC isolates' phylotypes, antibiotic resistance, and biofilm formation capacities is crucial for veterinary and public health.

This study aims to evaluate the phylotypes, antibiotic resistance, and biofilm formation potentials of EPEC isolates from diarrheic cats.

Material and Methods

Ethical Statement

This study was conducted with the approval of the Local Ethics Committee for Animal Experiments of Aydın Adnan Menderes University (ADÜ-HADYEK), dated 24.08.2023, numbered: 68583101/2023/130.

Material

This study utilized rectal swab samples from 37 diarrheic cats, received from a private clinic at Aydın Adnan Menderes University University Faculty of Veterinary Medicine Department of Microbiology Laboratories between September and December 2023. The feces of these cats, brought with complaints of diarrhea, were evaluated by the veterinarian during clinical examinations. The color of the stool generally varied from light brown to yellow, and its consistency was watery to semi-solid. Mucus and/or blood has been detected in some stool samples. The general condition of cats is characterized by loss of appetite, weakness and, in some, signs of dehydration. These cats, mostly mixed-breed, ranged in age from 2 months to 6 years. Cats that had not received antibiotic treatment were included in the study. Owners provided informed consent for their pets to participate. Fecal samples were collected using rectal swabs by a veterinarian, inserted approximately one cm into the anal sphincter and rotated to obtain visible fecal material. Samples were immediately placed in Stuart transport medium (Remel, USA) and stored at 4-8°C until inoculation [20].

Bacterial Isolation and Identification

Rectal swab samples were inoculated onto EMB agar (Merck, Germany) and aerobic conditions incubated at 37°C for 18-24 h. Metallic greenish shiny colonies were selected and subcultured onto MacConkey agar (Merck, Germany). After incubation, pink lactose-fermenting colonies were subcultured onto blood agar. Gram-negative rod morphology, lactose fermentation, negative oxidase, positive catalase and indole tests, and motility were considered presumptive for *E. coli* [21]. The isolates were preserved in Brain Heart Infusion Broth (Merck, Germany) with 20% glycerol at -20°C. Suspected *E. coli* isolates were molecularly identified by examining the *trpA* genes [11].

Biofilm Formation

Biofilm formation was assessed using the CRA method [18]. *E. coli* isolates were streaked onto CRA and incubated at 37°C for 24 h. Black, dry consistency colonies indicated positive biofilm production [18]. *E. coli* ATCC 25922 and *S. aureus* ATCC 25932 served as positive and negative controls for CRA method, respectively.

Antibiotic Susceptibility Tests

Antibiotic susceptibility tests were performed using the disk diffusion method according to CLSI (2020) [22]. Eight antibiotics from eight different families were tested: ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 20 µg/10 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), gentamicin (GEN, 10 µg), trimethoprim-sulfamethoxazole (SXT, 1.25 µg/23.75 µg), cefotaxime (CTX, 30 µg), meropenem (MEM, 10 µg) (Oxoid, United Kingdom) *(Table 1)*. *E. coli* ATCC 25922 served as the quality control strain. Isolates resistant to three or more antibiotic classes were classified as MDR [23].

Polymerase Chain Reaction

DNA was extracted using the InstaGene™ Matrix kit

Table 1. *Antibiotics used in the study, disk contents, evaluation criteria, and resistance statuses*

Antimicrobial Family	Antibiotic	Disc Content (µg)	≥S	≤R	Number of Resistant Isolates (%)
Penicillin	Ampicillin	10	17	13	24 (86)
Beta Lactam	Amoxicillin Clavulanic acid	20/10	18	13	21 (75)
Quinolones	Ciprofloxacin	5	26	21	20 (71)
Tetracycline	Tetracycline	30	15	11	18 (64)
Aminoglycoside	Gentamicin	10	15	12	17 (61)
Sulfonamide	Trimethoprim sulfamethoxazole	1.25/23.75	16	10	15 (54)
Cephem	Cefotaxime	30	26	22	14 (50)
Carbapenem	Meropenem	10	23	19	0 (0)

S: *Susceptible,* ***R:*** *Resistant*

Table 2. Primers used in the study

Primer	Target Gene	Sequence (5'-3')	Amplicon Size (bp)
EHEC	*stx*1	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	150
EHEC	*stx*2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255
EHEC/ EPEC	*eae*A	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384
EPEC	*bfp*A	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTA GT	300
*chu*A.1b *chu*A.2	*chu*A	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288
*yja*A.1b *yja*A.2b	*yja*A	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211
*TspE*4C2.1b *TspE*4C2.2b	*TspE*4.C2	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	152
*Ace*K F *Arp*A1 R	*arp*A	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400
*Arp*AgpE F *Arp*AgpE R	*arp*A	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301
*trp*AgpC.1 *trp*AgpC.2	*trp*A	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCCC	219
*trp*BA.F *trp*BA.R	*trp*A	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489

(Biorad, Dubai) per the manufacturer's instructions. Purity and quantity were assessed via a nanodrop spectrophotometer (Maestrogen, Taiwan), with an OD260/OD280 ratio of 1.6-2.0 [24]. Primers targeting *stx1*, *stx2*, *eaeA* genes identified EHEC [25], while *eaeA* and *bfpA* genes identified EPEC [26] *(Table 2)*. Phylogenetic distribution was determined using the PCR method [11,27-29] *(Table 2)*.

PCRs were performed in 25 µL volumes with final concentrations: 1x Taq enzyme buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 ρmol primers, and 1.5 U Taq DNA polymerase (Fermentas, USA). PCR tubes were prepared with 22 µL of master mix and 3 µL of DNA for each sample. Amplification involved initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, annealing at 55°C *(stx1, stx2, eaeA, bfpA)* and 56°C *(chuA, yjaA, tspE4.C2, arpA, trpA)* for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. *E. coli* ATCC 35150 (EHEC; *stx1, stx2, eaeA)* served as the positive control, and *S.* Typhimurium ATCC 14028 as the negative control. Target genes producing a single band of the expected size upon amplification were considered positive.

Statistical Analysis

For statistical analysis, Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS Inc., USA) was used. Pearson's chi-square ($\chi 2$) test (Fisher's Exact $\chi 2$ Test) compared frequency data. The relationship between isolate pathotypes and biofilm formation, phylotype, and MDR status was evaluated using the $\chi 2$ test. Results were evaluated at a 95% confidence interval and with $P<0.05$ were considered statistically significant.

Results

Isolation and Identification

E. coli was isolated in 75% (28 isolates) of rectal swab samples taken from 37 cats with diarrhea. Molecular confirmation via PCR targeting the *trpA* gene verified all isolates as *E. coli*. Subsequent analysis included evaluation of pathotypes, phylotypes, biofilm formation potentials, and antibiotic resistance profiles of the isolates.

Pathotyping

Among the 28 *E. coli* isolates, 46% (13 isolates) were classified as EPEC isolates. The prevalence of tEPEC was found to be 18% (5 isolates) (both *eaeA* and *bfpA* positive), and the prevalance aEPEC was 28% (8 isolates) (*eaeA* gene positive). There was no EHEC pathotype among the isolates *(Fig. 1)*.

Phylotyping

Using available primers, 93% (26 isolates) of *E. coli* isolates were phylotyped. Among these, 22% were phylogroup B2, 18% B1, 18% C, 14% D, 11% A, 7% E, and 3% F. The phylotype of two isolates (7%) could not be determined *(Fig. 2)*.

Table 3. *Phylotypes of E. coli isolates*	
Phylogroups	**Isolate number (n=28) (%)**
A	3 (11)
B1	5 (18)
C	5 (18)
E	2 (7)
B2	6 (22)
D	4 (14)
F	1 (3)
?	2 (7)

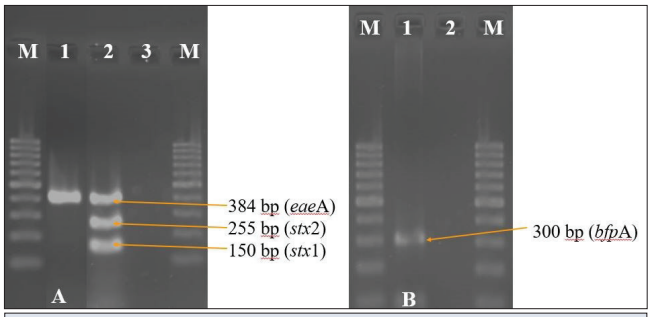

Fig 1. Agarose gel electrophoresis of virulence gene PCR products associated with pathotype. A.1. *eaeA* gene (384 bp) 2. Positive Control EHEC (*E. coli* ATCC 35150, *stx1*:150 bp, *stx2*: 255 bp, *eaeA*: 384 bp) 3. Negative Control (S. Typhimurium ATCC 14028) M: 100 bp DNA Ladder (Fermentas, USA). B.1. *bfpA* gene (300 bp) 2. Negative Control (S. Typhimurium ATCC 14028) M: 100 bp DNA Ladder (Fermentas, USA)

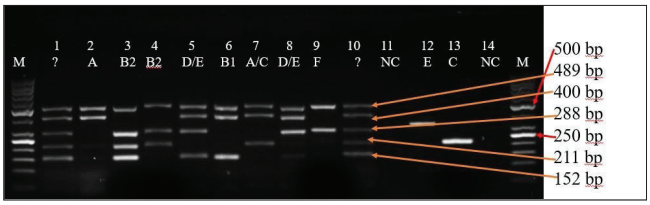

Fig 2. PCR profiles with the new Clermont phylotyping method. 1. Unknown phylogroup (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), 2. Phylogroup A (– – – + +), 2. Phylogroup B2 (+ + + – +), 3. Phylogroup B2 (– + + – +), 5. Phylogroup D/E (+ – + + +), 6. Phylogroup B1 (+ – – ++), 7. Phylogroup A/C (– + – + +), 8. Phylogroup D/E (– – + + +), Group F (– – + – +) 10. Unknown phylogroup (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), 11. Negative Control (NC): DNA-free master mix 12. Phylogroup E (301 bp) 13. Phylogroup C (219 bp) 14. NC: S. Typhimurium ATCC 14028 M: Marker (50 bp) (Fermentas, USA), ?: Isolates whose phylotype could not be determined

In cat diarrhea cases, the most common phylogroup was B2 (22%), and the least observed was F (3%). Commensal phylogroups (A, B1, C, E) comprised 54% (15 isolates), while pathogenic phylogroups (B2, D, F) comprised 39% (11 isolates) *(Table 3, Fig. 3)*.

Biofilm Formation

Of the 28 clinical *E. coli* isolates, 39% (11 isolates) were determined to form biofilm *(Fig. 4)*.

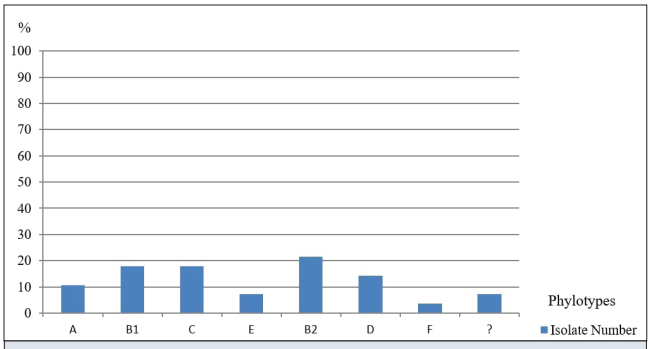

Fig 3. Distribution of *E.* coli phylotypes (n=28)

Antimicrobial Resistance

The antimicrobial resistance profiles of *E. coli* isolates varied: most were resistant to ampicillin (86%), followed by amoxicillin-clavulanate (75%), ciprofloxacin (71%), tetracycline (64%), gentamicin (61%), trimethoprim-sulfamethoxazole (54%), and cefotaxime (50%). No isolates were resistant to meropenem *(Table 1, Fig. 5)*.

Multiple Antibiotic Resistance

Eighty-six percent of *E. coli* isolates exhibited MDR, while 15% were non-multidrug resistant (NMDR). Multiple antibiotic resistance status and antibiotic resistance phenotypes of isolates are shown in *Table 4, Fig. 6.*

Statistical Results

Our study found no significant relationship between the pathotype of *E. coli* isolates and their biofilm formation or MDR status. However, a significant relationship was identified between the pathotype and phylotype B2. No significant relationships were found between the other phylotypes (A, B1, C, D, E, F) and the pathotype *(Table 5)*.

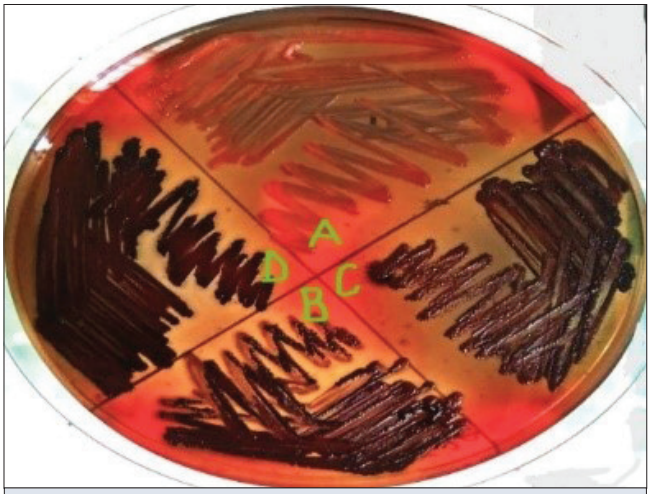

Fig 4. Biofilm-forming and non-biofilm-forming isolates on Congo Red Agar. A: Negative Control (*S. aureus* ATCC 25932), B: Positive Control (*E. coli* ATCC 25922), C, D: Biofilm-forming *E. coli* isolates

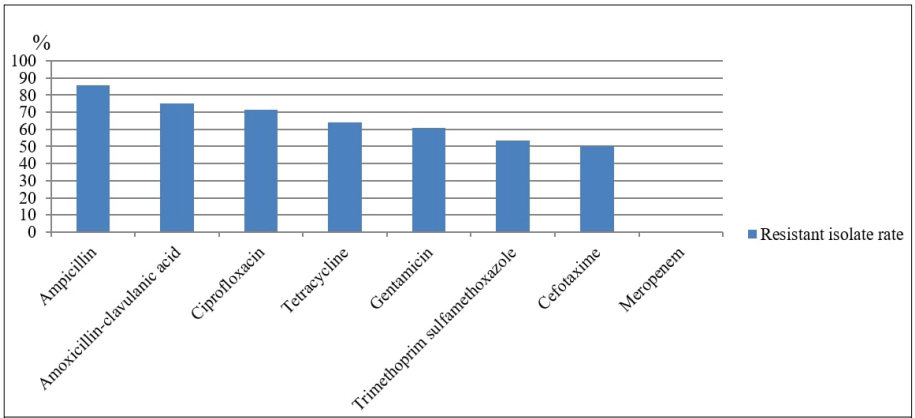

Fig 5. Resistance rates of *E. coli* isolates to different antibiotics

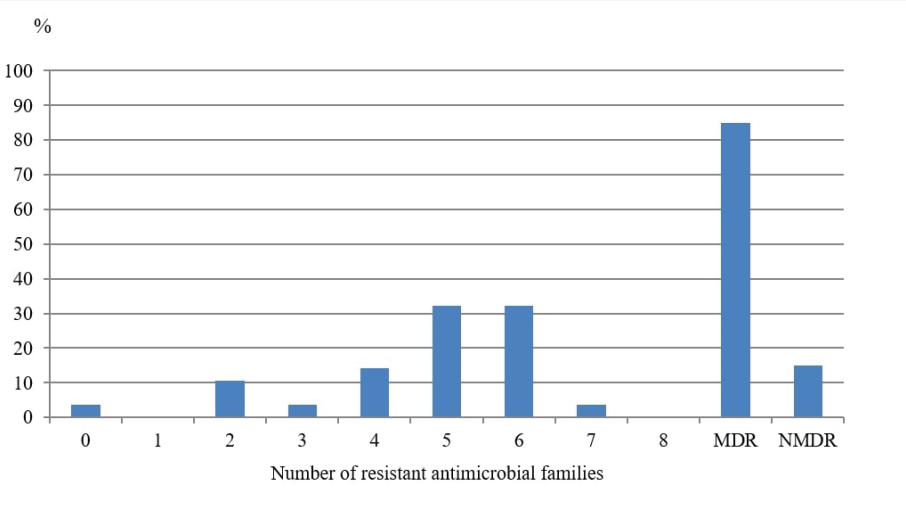

Fig 6. MDR/NMDR resistance rates of *E. coli* isolates (n=28).

Table 4. *Multiple antibiotic resistance status of isolates*

Antibiotic Resistance Phenotype (Number of Isolates)	**Number of Isolates (n=28) (%)**	**MDR/NMDR status**
Beta Lactam, Sulfonamid (1) Tetracycline, Aminoglycoside (1) Aminoglycoside, Cephem (1)	3 (11)	NMDR 4 (15)
Penicillin, Beta Lactam, Quinolones (1)	1 (4)	MDR 24 (85)
Penicillin, Quinolones, Aminoglycoside, Sulfonamide (1) Penicillin, Beta Lactam, Tetracycline, Aminoglycoside (1) Penicillin, Beta Lactam, Tetracycline, Sulfonamide (1) Penicillin, Beta Lactam, Aminoglycoside, Sulfonamide (1)	4 (15)	
Penicillin, Beta Lactam, Quinolones, Tetracycline, Sulfonamide (4) Penicillin, Quinolones, Aminoglycoside, Sulfonamide, Cephem (1) Penicillin, Quinolones, Tetracycline, Aminoglycoside, Cephem (1) Penicillin, Beta Lactam, Quinolones, Sulfonamide, Cephem (2) Penicillin, Beta Lactam, Quinolones, Tetracycline, Cephem (1) Penicillin, Beta Lactam, Quinolones, Aminoglycoside, Cephem (1)	10 (34)	
Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Cephem (5) Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide (2) Penicillin, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide, Cephem (1)	8 (28)	
Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide, Cephem (1)	1 (4)	

MDR: *Multi-Drug Resistance,* ***NMDR:*** *Non Multi Drug Resistance*

Table 5. *Relationship between biofilm formation, multiple antibiotic resistance and phylotypes of enteropathogenic and non-enteropathogenic E. coli isolates*

Biofilm formation, MDR, Pylotypes Characteristics of Isolates	Pathotype		χ2	P Value
	EPEC (n=13)	NEPEC (n=15)		
Biofilm (+)	7	4	2.16	0.246
Biofilm (-)	6	11		
MDR (+)	12	12	0.83	0.600
MDR (-)	1	3		
Phlotype A (+)	1	2	0.22	1
Phlotype A (-)	12	13		
Phlotype B1 (+)	2	3	0.09	1
Phlotype B1 (-)	11	12		
Phlotype B2 (+)	6	0	8.50	**0.005****
Phlotype B2 (-)	7	15		
Phlotype C (+)	2	3	0.09	1
Phlotype C (-)	11	12		
Phlotype D (+)	2	2	0.02	1
Phlotype D (-)	11	13		
Phlotype E (+)	0	2	1.80	0.484
Phlotype E (-)	13	13		
Phlotype F (+)	0	1	0.86	1
Phlotype F (-)	13	14		

MDR: *Multi-Drug Resistance,* ***EPEC:*** *Enteropathogenic Escherichia coli,* ***NEPEC:*** *Non-Enteropathogenic Escherichia coli*

Discussion

Pet ownership is increasing, yet pets can harbor zoonotic diseases, posing public health risks [7,20,30]. Previous research suggests that *E. coli* is a cause of diarrhea in cats [19,30,31]. Characterizing EPEC isolates from diarrheic cats and assessing their antibiotic resistance are crucial for veterinary and public health. This study aims to determine the diversity and pathogenic properties of *E. coli* in cats, enhancing understanding of potential zoonotic risks from pets to humans.

EPEC has been documented in cats with diarrhea, with prevalence rates of 18% in the United States [32], 2.5% in Brazil [19]. In this study, a higher prevalence of EPEC (46%) was observed. The variability in EPEC prevalence cannot be ascribed to a singular factor. Environmental influences, climate, and living conditions of pets significantly impact the spread and prevalence of microorganisms. Regional prevalence differences may stem from these geographical disparities. Furthermore, variations in sample sizes and selection criteria across studies can influence prevalence rates. The diagnostic methods employed to detect EPEC also contribute to differing results. Additionally, the general health, feeding habits, and care conditions of cats are crucial factors, with infections being more prevalent in immunocompromised cats.

Our study found EPEC prevalence in diarrheic cats to be 46%, with 28% attributed to aEPEC and 18% to tEPEC. Previous research has reported EPEC cases in cats [19,20,33], some indicating the presence of the *bfpA* gene, associated with tEPEC [34,35]. Similarly, our study detected tEPEC, but aEPEC prevalence was higher, consistent with other studies [19]. However, EHEC pathotype detection was negative in our study, aligning with certain researchers' findings [19] but conflicting with others [30]. We speculate that other pathotypes like EHEC may exist in diarrheic cats, but our limited sample size may have precluded a definitive conclusion.

In this study, only the EHEC pathotype, aside from EPEC, was examined. The EHEC pathotype is crucial for assessing zoonotic risks that can be transmitted from cats to humans and poses a significant threat to human health. While other pathotypes are also important, the focus on EHEC was due to limited resources and time, prioritizing this pathotype for its high risk to human health. We used the CRA method to assess biofilm formation, which is cost-effective and rapid [17,18]. *E. coli* utilizes extracellular curli for adhesion and biofilm formation [36]. Biofilm

formation is a fundamental bacterial survival mechanism and their default lifestyle [14]. In a previous study, *E. coli* isolates obtained from calves with diarrhea were reported to be strong biofilm producers (62.5%) by the CRA method [37]. In this study, it was observed that EPEC isolates had a significant biofilm formation potential in the CRA method. Although the CRA method is widely used and provides useful information; may not capture the full complexity of biofilm formation compared to other methods (microplate, electron microscopy, etc.). Therefore, we acknowledge the limitations of using CRA alone and recommend using complementary methods to provide a more comprehensive assessment of biofilm formation. We found 39% of all isolates and 54% of EPEC isolates formed biofilms, suggesting a heightened biofilm production potential in EPEC isolates. Additionally, all seven biofilm-forming EPEC isolates were MDR, consistent with other studies [38], highlighting the importance of understanding the link between antimicrobial resistance and biofilm formation in EPEC isolates. However, when the data were analyzed in detail, we could not detect a significant relationship between the pathotype of the *E. coli* isolates in our study and their ability to form MDR or biofilm. This result may be due to the small sample size, which limits the statistical power to detect associations. Additionally, genetic variation and environmental factors such as prior antibiotic exposure and changing conditions that influence biofilm formation may also contribute to this lack of correlation.

The genomic structure of *E. coli* suggests that different phylogroups may correlate with disease status and isolation source [11]. It has been reported in previous studies that the B2 phylogroup can be found in both intestinal and extraintestinal pathogenic *E. coli* species [39]. In this study, phylogroup B2 was detected as the most common phylogroup in intestinal EPECs. These phylogroups are crucial in determining *E. coli*'s pathogenic characteristics and infection risks. Limited research exists on *E. coli* pathotypes in cats globally [19]. Prior studies on EPEC pathotypes in companion animals did not assess *E. coli*'s phylogenetic grouping [19,40]. A study in Brazil reported higher EPEC prevalence in pets with diarrhea, classified into phylogroups B1 and E [33]. Our study identified diverse phylotypes (B2, B1, C, D, A, E, F), highlighting genetic diversity in *E. coli* isolates from cats. It is important to consider that differences in phylogroup distribution may occur as a result of a combination of multiple factors (geographical factors, sampling methods, environmental conditions, diagnostic techniques used, etc.).

In this study, the phylogenetic group of two *E. coli* isolates that did not form biofilms could not be determined. Notably, one of these isolates exhibited MDR, while the other was susceptible to all tested antibiotics. This suggests significant genetic diversity among *E. coli* isolates associated with diarrheal cases, indicating potentially distinct virulence properties.

The rise of MDR *E. coli* is a global public health concern due to its opportunistic pathogenic nature [7]. Daily interactions between pets and their owners facilitate the sharing of *E. coli* strains, highlighting the need to monitor antimicrobial resistance in *E. coli* from domestic animals [7,20,30]. Our study found antibiotic-resistant EPEC strains in the intestines of cats, with resistance levels between 50% and 86% for all tested antibiotics except meropenem. Additionally, 86% of isolates exhibited multiple antibiotic resistance. However, the small sample size limits the generalizability of these findings.

In this study, we evaluated *E. coli* resistance profiles to eight antibiotics using the disc diffusion method. Ampicillin resistance was most prevalent at 86%, followed by 75% for amoxicillin-clavulanic acid, consistent with findings from Bangladesh [30]. These results are clinically significant as broad-spectrum antibiotics like penicillin and β-lactams are frequently used for gastrointestinal issues in pets [41]. Notably, antibiotic-resistant *E. coli* can persist in dogs' intestines for up to 21 days post-treatment, highlighting the gut as a reservoir for resistant bacteria [42]. Resistance genes can persist for years after antibiotic exposure [43]. While resistance was detected to seven antibiotics tested in this study, no resistance was detected to meropenem. Meropenem is an antibiotic generally used in human medicine as a last resort effective against Gram-negative bacteria. The lack of meropenem resistance can be explained by both clinical usage habits and the genetic and biological characteristics of the isolates. In a study from Bangladesh, cefotaxime resistance was 100%, compared to 50% in our study, likely due to less frequent use of third-generation cephalosporins in our sampled cats [30]. Resistance rates for trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, gentamicin, and ciprofloxacin were also lower than those reported in Egypt [44]. Our findings showed that 86% of *E. coli* isolates were MDR, compared to 49% in a recent Chinese study [45]. These differences underscore how antibiotic usage and veterinary policies influence resistance rates in different regions.

Our study identified a significant correlation between *E. coli* pathotype and the B2 phylotype, which is commonly associated with ExPEC strains that cause various infections in animals and humans. The significant relationship between EPEC pathotype and B2 phylotype may indicate that these EPEC isolates may have high pathogenic potential. The B2 phylotype is generally associated with more virulent *E. coli* isolates [39]. This is important to highlight the seriousness of EPEC infections in cats and possible zoonotic risks. In contrast, no significant relationship was found between other phylotypes (A, B1,

C, D, E, F) and the EPEC pathotype, indicating that these phylotypes may not independently determine pathogenic properties.

Two isolates carrying all the genes examined in this study were identified. However, these two isolates could not be phylotyped with the primers available in the method used [11]. This indicates that these two isolates may be a new phylotype that cannot be detected with existing primers. To more precisely determine the genetic diversity and phylogenetic relationships of *E. coli* isolates, more comprehensive and detailed analysis methods such as Multi-Locus Sequence Typing (MLST) need to be applied.

In conclusion, our findings offer valuable insights into the prevalence, phylogroup distribution, biofilm formation potential, and antibiotic resistance of EPECs isolated from cats with diarrhea. The noteworthy association between the EPEC pathotype and the B2 phylotype suggests a potential risk posed by these isolates.

Declarations

Availability of Data and Materials: The corresponding author (H. T. Yüksel Dolgun) can provide the datasets of this research upon reasonable request.

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Author Contributions: HTYD, YS, ST: Conceptualization, Methodology, Validation, Analysis, Investigation, Writing-Original Draft, Editing. All authors read and approved the final manuscript.

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Research Article

Mitochondrial Fusion Protein 2 Affects Intracellular Survival of *Brucella abortus* A19 by Regulating Endoplasmic Reticulum Stress and Apoptosis

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Abstract

Mitochondrial fusion protein 2 (MFN2) deficiency has been shown to hinder the survival of bacteria in macrophages. Endoplasmic reticulum stress and apoptosis are vital defense mechanisms against Brucella infection, but the specific role of MFN2 in Brucella-infected macrophages remains unclear. In this study, we aimed to investigate the role of MFN2 in the infection of macrophages by Brucella abortus strain A19. The levels of CHOP and GRP78, which are molecules associated with endoplasmic reticulum stress, as well as Caspase-3 and BAX, which are pro-apoptotic molecules, were measured using confocal microscopy, qRT-PCR and Western blot in cell models infected with B. abortus A19. Additionally, the apoptosis rate of these cell models was assessed using flow cytometry. Our findings revealed a significant decrease in MFN2 levels 24 h post B. abortus A19 infection of macrophages. Interfering with MFN2 in macrophages led to an increase in Brucella-induced up-regulation of CHOP, GRP78, Caspase-3, and BAX, consequently hindering the survival of B. abortus A19 in macrophages. Conversely, infecting macrophages that overexpress MFN2 with B. abortus A19 resulted in the down-regulation of CHOP, GRP78, Caspase-3, and BAX. MFN2 mediated the down-regulation of endoplasmic reticulum stress and programmed cell death in B. abortus A19-infected macrophages, thereby supported the intracellular survival of Brucella. This is the first report to highlight the key role of MFN2 in the intracellular survival of Brucella, providing a new perspective for understanding the mechanisms involved and offering a potential research direction for the development of targeted therapeutic agents against brucellosis.

Keywords: *Brucella abortus* A19, Mitochondrial fusion protein 2, Endoplasmic reticulum stress, Apoptosis, Intracellular survival

Introduction

Brucellosis is a zoonotic disease caused by *Brucella* species. It is a significant animal-borne disease that is widespread in over 170 countries and regions worldwide. In livestock, it commonly presents as a reproductive disorder, while in humans, it manifests as a chronic febrile illness. Due to its persistent nature and widespread prevalence, the disease is challenging to eliminate and poses a significant threat to both animal husbandry and human health [1-3].

The endoplasmic reticulum (ER) is a crucial organelle that plays a role in maintaining cellular homeostasis [4]. It synthesizes intracellular proteins and lipids and has close interactions with mitochondria, allowing for signaling and substance exchange. Mitochondrial fusion protein 2 (MFN2) is a GTPase protein found in the outer mitochondrial membrane and the mitochondria-associated membrane. It acts as a bridge between mitochondria and the endoplasmic reticulum, and its absence leads to endoplasmic reticulum stress. MFN2 also regulates mitochondrial fusion and the transfer of calcium from the endoplasmic reticulum to mitochondria [5]. Numerous studies have demonstrated the crucial role of MFN2 in the innate immune response during pathogenic infections [6-8]. Previous studies have shown that MFN2 inhibits the antiviral immune response in hepatitis B virus-associated hepatocellular carcinoma [6].

In HIV-1 Vpr-infected HEK293 cells, overexpression of MFN2 reduces cell death by facilitating interactions between the endoplasmic reticulum and mitochondria [7]. Additionally, in *Mycobacterium tuberculosis*-infected THP-1 macrophages, MFN2 is involved in the assembly and activation of NLRP3 inflammatory vesicles during infection [8].

Brucella, an intracellular parasitic bacterium, employs various strategies to invade host cells. It primarily targets specialized phagocytes like macrophages, dendritic cells, as well as non-specialized phagocytes such as fibroblasts and trophoblasts. Once inside the host cells, *Brucella* releases effector molecules into the host cytoplasm. This facilitates *Brucella*'s transport to the endoplasmic reticulum (ER), resulting in significant reorganization of the ER and disruption of its homeostasis. These events induce ER stress, triggering the unfolded protein response (UPR), which aids in the survival and establishment of proliferative compartments [9,10]. In the context of ER stress, the transcription factor CHOP plays a crucial role. If the ER stress persists and overwhelms the capacity of the cellular response, it leads to increased expression of CHOP. In turn, CHOP acts as a major pro-apoptotic transcription factor, ultimately driving the cells towards apoptosis [11,12]. Glucose-regulated protein (GRP78) is a key chaperone protein located in the endoplasmic reticulum (ER). It is synthesized in response to various stress conditions that disrupt ER function and homeostasis [13]. *Brucella* has been shown to regulate apoptosis [14,15]. Inhibition of apoptosis in infected cells supports the intracellular replication of *Brucella* [15], with mitochondrial division being a crucial step in the apoptotic process. The pro-apoptotic protein BAX, a member of the Bcl-2 family, is recruited to the outer mitochondrial membrane during apoptosis, leading to mitochondrial fragmentation and blocking of mitochondrial fusion [16]. BAX, which is normally distributed in cytoplasmic lysates, plays a role in regulating mitochondrial morphology [17]. *Brucella* has been found to interact with mitochondria in host cells, a process that is essential for intracellular recycling and infection of neighboring cells by *Brucella* [10]. Caspase-3 is also an important marker of apoptosis. *Brucella* infection of RAW264.7 cells for 48 h can induce apoptosis by upregulating the expression of caspase-3 via the effector protein BtpB [15].

The role of MFN2 in *Brucella* infected host cells has not been investigated. To investigate the role of MFN2 during Brucella infection of cells, MFN2 was found to mediate the down-regulation of endoplasmic reticulum stress and programmed death in *B. abortus* A19-infected macrophages and to support the intracellular survival of *B. abortus* A19. Our study provides a theoretical basis for further investigation of the MFN2 gene and the pathogenesis of *Brucella*. In addition, the MFN2 gene may be a potential target for the future development of brucellosis prevention and therapeutic approaches.

Materials and Methods

Bacterial Strain and Cell Line

B. abortus A19 was purchased from the Xinjiang Tiankang Animal Biotechnology Co. Ltd., China. *B. abortus* was cultured on Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) (Oxoid, UK). The culture conditions in TSB were aerobic conditions at 37°C, 170 r/min for 3-4 d. *Escherichia coli* strains DH5α and BL21 were used to transform the PCDNA3.1-EGFP-MFN2 overexpression recombinant plasmid and were cultured on Luria-Bertani (LB) medium (Oxoid, UK) under aerobic conditions at 37°C and 170 r/min for 12 h. The mouse macrophage RAW264.7 line (RAW264.7, TIB-71) cell line (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37°C with 5% CO_2. All research work on *B. abortus* A19 was conducted in a biosafety level 3 laboratory.

Construction of MFN2 Interference and Overexpression Cell Models

The mRNA sequences of CHOP, GRP78, Caspase-3, BAX, MFN2 and GAPDH genes of mice published on the NCBI website GenBank were retrieved and primers were designed using Primer 5.0 *(Table1)*. The siRNA interference fragments (siMFN2-2275, siMFN2-450, siMFN2-1661) were synthesized by Anhui General Biological Company (Anhui, China) *(Table 2)*. The PCDNA3.1-EGFP plasmid (Anhui General Bio, China) was digested by Hind III and BamH I and ligated with MFN2 gene to construct the PCDNA3.1-EGFP-MFN2 recombinant plasmid.

Table 1. *Primer sequence of qRT-PCR*

Gene	Primer Sequence (5'→3')
CHOP-F	5'-CTGGAAGCCTGGTATGAGGAT-3'
CHOP-R	5'-CAGGGTCAAGAGTAGTGAAGGT-3'
GRP78-F	5'-ACTTGGGGACCACCTATTCCT-3'
GRP78-R	5'-ATCGCCAATCAGACGCTCC-3'
Caspase-3-F	5'-GAGCTTGGAACGCGAAGAAA-3'
Caspase-3-R	5'-TTGCGAGCTGACATTCCAGT-3'
BAX-F	5'-TGAAGACAGGGGCCTTTTTG-3'
BAX-R	5'-AATTCGCCGGAGACACTCG-3'
MFN2-F	5'-CCAACTCCAAGTGTCCGCTC-3'
MFN2-R	5'-GTCCAGCTCCGTGGTAACATC-3'

Table.2. Interference sequence of MFN2 siRNA

Name	Sequence (5'→3')
siMFN2-450-F	5'-ACACAUGGCUGAAGUGAAUTT-3'
siMFN2-450-R	5'-AUUCACUUCAGCCAUGUGUTT-3'
siMFN2-1661-F	5'-CGGAGGAAGUGGAAAGGCATT-3'
siMFN2-1661-R	5'-UGCCUUUCCACUUCCUCCGTT-3'
siMFN2-2275-F	5'-GCAGUGGGCUGGAGACUCATT-3'
siMFN2-2275-R	5'-UGAGUCUCCAGCCCACUGCTT-3'
siMFN2-Negative-F	5'-UUCUCCGAACGUGUCACGUTT-3'
siMFN2-Negative-R	5'-ACGUGACACGUUCGGAGAATT-3'

Interference and overexpression systems were constructed by transfecting murine macrophage RAW264.7 cells with interference fragments and recombinant plasmids using Advanced DNA RNA Transfection Reagent (Zeta Life, USA).

Detection of Interference Efficiency of siRNA on MFN2

RAW264.7 cells were transiently transfected with siMFN2-2275, siMFN2-450, siMFN2-1661, and siMFN2-Negative interfering sequences at concentrations of 10 nM/μL as described previously [18], and cellular RNA and total protein were collected 48 h after transfection. Subsequently, the RNA was reverse transcribed into cDNA followed by the use of qRT-PCR to detect the mRNA expression level of MFN2. Cellular proteins were collected and the MFN2 protein expression level was assessed using Western blot analysis.

Interference efficiency = (siRNA-negative control group - siRNA experimental group)/siRNA - negative control group × 100%

Detection of Overexpression Efficiency of PCDNA3.1-EGFP-MFN2 Recombinant Plasmid

The PCDNA3.1-EGFP-MFN2 recombinant plasmid at a concentration of 6 μg was transiently transfected into RAW264.7 cells as described previously [19], and RNA and total protein were collected from the cells at 48 h of transfection. Then, the RNA was reverse transcribed into cDNA, and the mRNA expression level of MFN2 gene was detected by qRT-PCR, and the protein expression level of MFN2 was detected by Western blot [19].

Detection of *B. abortus* A19-Induced CHOP, GRP78, Caspase-3, BAX Expression Levels After MFN2 Interference and Overexpression

Interfering cells and overexpressing cell models were infected with *B. abortus* A19 respectively, and cellular RNA and total protein were collected at 24 h post-infection. Expression levels of endoplasmic reticulum stress signature molecules CHOP and GRP78 were detected by confocal microscopy. qRT-PCR and Western blot were used to detect the mRNA transcript and protein expression levels of endoplasmic reticulum stress signature molecules CHOP, GRP78 and pro-apoptotic genes Caspase-3 and BAX.

Detection of Apoptosis Induced by *B. abortus* A19 After MFN2 Interference and Overexpression

B. abortus A19 was used to infect the MFN2-interfering cell model and overexpressing cell model, respectively. Cells were collected at 24 h post-infection, digested with EDTA-free trypsin, adjusted to a cell concentration of 1-5x10^5/mL, and centrifuged at 800 rpm for 5 min before discarding the supernatant, and the cells were washed twice using PBS, and then processed in accordance with the apoptosis assay kit (Absin, China), and then immediately detected by flow cytometry.

Intracellular Survival of *B. abortus* A19 After MFN2 Interference and Overexpression

The best interfering and overexpressing cell models were infected with *B. abortus* A19 respectively, and the cells were lysed with 0.02% Triton X-100 at 0, 6, 12, and 24 h post-infection, and the lysates were coated in *B. abortus* solid medium and assayed for intracellular survival of *Brucella* by CFU counting with reference to the experimental method of Zhang et al.[20].

Confocal Microscopy Inspection

After 24 h of infection with *B. abortus* A19 in interfering and overexpressing cell models, cells were fixed on coverslips using 4% paraformaldehyde for 15 min. Subsequently, coverslips were rinsed thrice with PBS, and cells were treated with 0.2% Triton X-100 for 5 min. After another round of rinsing with PBS, cells underwent blocking in a blocking solution for 1 h. The primary antibody was then diluted with PBST and incubated in a dark, humid environment for 1 h at 4ºC. Samples were incubated overnight, followed by washing thrice with PBST. The secondary antibody was diluted with PBST and samples were incubated for 1 h at room temperature in a dark, humid environment. After another round of washing with PBST, samples were sealed on slides using a DAPI-containing sealing agent, and finally examined under a fluorescence microscope.

qRT-PCR Detection

After infection of the interfering and overexpressing cell models by *B. abortus* A19, total RNA from the cells was collected and reverse transcribed to cDNA, and GAPDH was used as an internal reference, using the SYBR Green Master Mix kit (Roche, Switzerland) on QuantStudio 3 (ThermoFisher, USA). Real-time

fluorescent quantitative PCR was performed, and the 2-ΔΔCt method was used to calculate the relative expression of CHOP, GRP78, Caspase-3, BAX and MFN2 mRNAs.

Western Blot Detection

B. abortus A19 infected cells were washed 3 times with PBS, lysed on ice for 10 min by adding lysis solution (1mLRIPA+10 μLPMSF), the lysate was collected and total protein was collected by centrifugation at 4ºC, 12000 rpm for 5 min, and the protein was adjusted by BCA protein quantitative assay kit (ThermoFisher, USA). The concentration of protein was adjusted by BCA protein quantification kit (ThermoFisher, USA), separated by 12% SDS-PAGE and transferred to nitrocellulose (NC) membrane, closed with closure solution (5% skimmed milk powder in TBST) for 2 h at 37ºC, washed 3 times with PBS and incubated with primary antibody diluted with TBST overnight at 4ºC, washed 3 times with TBST and incubated with secondary antibody diluted with TBST at room temperature for 2 h. The membrane was washed with TBST 3 times and then stained with an ECL kit (ThermoFisher, USA).

Statistical Analysis

Data on intracellular CFU of *Brucella* were transformed into logarithms. The experimental data were analysed by One-Way ANOVA and 2 way ANOVA using SPSS 25.0 software (International Business Machines Corporation, USA), and the results were expressed as mean ± standard deviation, with $P<0.05$ considered statistically significant. GraphPad Prism 7.0 software (GraphPad Software, USA) was used to plot the data. All experiments were independently performed at least three times.

Results

B. abortus A19 Infection Induces a Decrease in Macrophage MFN2 Expression

Cells were collected at 6 h, 12 h and 24 h after *B. abortus* infection and the expression level of MFN2 was analysed by qRT-PCR and Western blotting. The results showed that the mRNA transcript level ($P=0.0047$) and protein expression level ($P=0.028$) of MFN2 were significantly reduced 24 h after *B. abortus* A19 infection of RAW264.7 cells, indicating that *B. abortus* A19 induced a decrease in macrophage MFN2 expression over time *(Fig. 1)*.

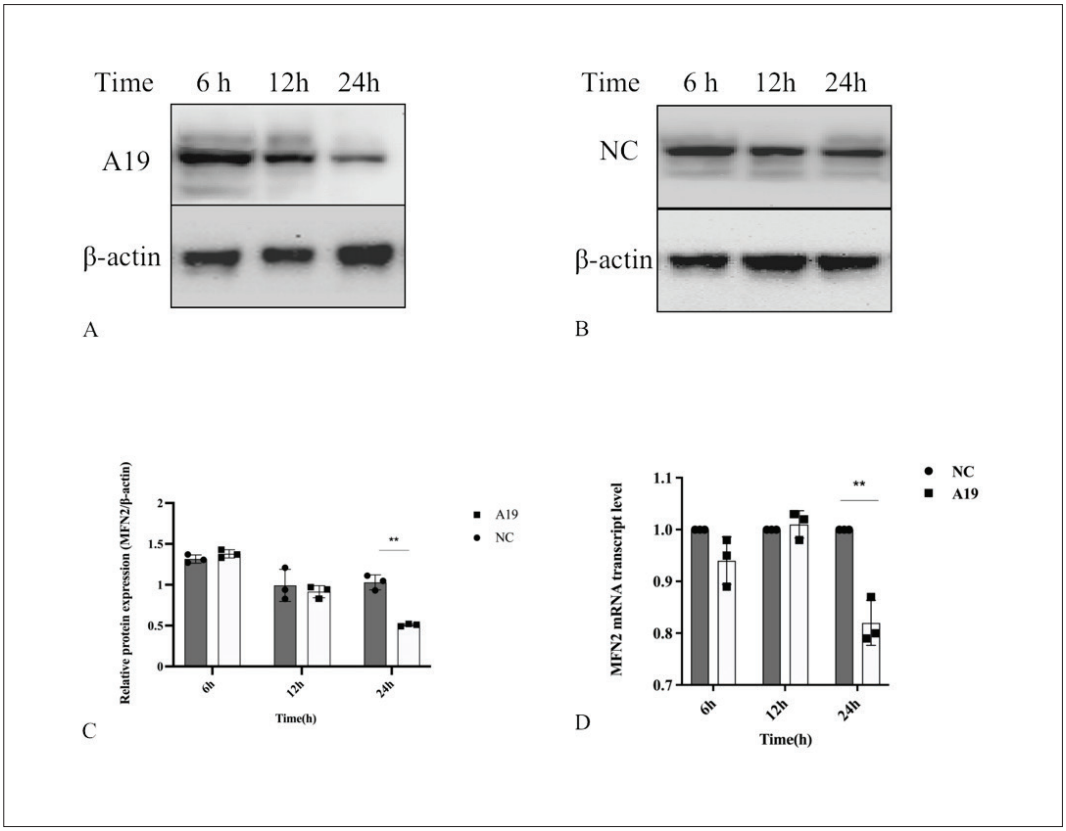

Fig 1. Detection of MFN2 mRNA and protein expression levels after *B. abortus* A19 infection. (A) Levels of MFN2 protein expression in RAW264.7 after infection with *B. abortus* A19 for 6, 12 and 24 h, (B) Negative control RAW264.7 cells MFN2 protein expression level after 6, 12 and 24 h (C), The ratio of cleaved MFN2 relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD, (D) MFN2 mRNA expression levels were detected by qRT-PCR after 6 h, 12 h, and 24 h of *Brucella* abortus A19 infection in RAW264.7 cells. *$P<0.05$; **$P<0.01$

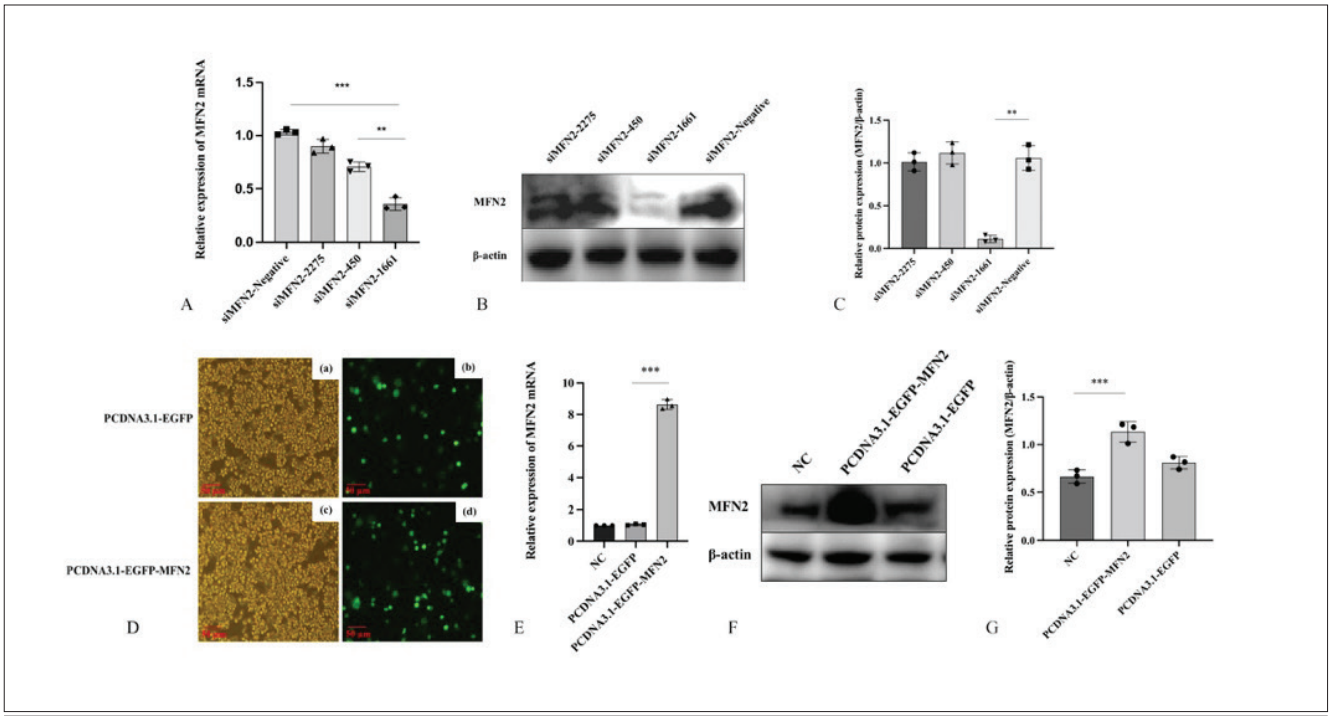

Fig 2. MFN2 overexpression and interference cell model construction. (A) qRT-PCR screening for the best interfering fragments of MFN2, (B) Western blot screening for the best interfering fragments of MFN2, (C) The ratio of cleaved MFN2 relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD, (D) Transfection efficiency of PCDNA3.1-EGFP-MFN2 overexpression recombinant plasmid by fluorescence microscopy. (a, b: PCDNA3.1-EGFP transfection of RAW264.7 in bright and dark field view, b, c: PCDNA3.1-EGFP-MFN2transfection of RAW264.7 in bright and dark field view), (E) Efficiency of MFN2 gene overexpression detected by qRT-PCR, (F) Efficiency of MFN2 gene overexpression detected by Western blot, (G) The ratio of cleaved MFN2 relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD. **P<0.01; ***P<0.001

Infection and Overexpression Cell Model Construction

The optimal interfering sequence was screened using qRT-PCR and Western blotting. The results showed that siMFN2-1661 had the highest inhibition rate (P<0.001) of MFN2 mRNA compared with the control group (siMFN2-Negative group) *(Fig. 2-A,B,C)*. This suggests that siMFN2-1661 is the best interfering fragment for constructing the MFN2 gene interference cell model.

After transfection of the recombinant plasmid PCDNA3.1-EGFP-MFN2 into RAW264.7 cells for 48h, a large amount of green fluorescent protein expression was observed by inverted fluorescence microscopy *(Fig. 2-D)*, indicating that the overexpression recombinant plasmid was successfully transfected. qRT-PCR results showed that compared with the control group (PCDNA3.1-EGFP group), the overexpression group (PCDNA3.1-EGFP-MFN2 group) had a significantly higher expression level of MFN2 mRNA than the control group, and the expression amount was about 8 times higher than that of the non-overexpressed group (PCDNA3.1-EGFP group) (P<0.001) *(Fig. 2-E)*. The Western blotting results showed that the expression level of MFN2 protein in the overexpression group was significantly higher than that in the control group (P<0.001) *(Fig. 2-F,G)*, indicating that the MFN2 gene overexpression cell model was successfully constructed.

B. abortus A19 Infection of MFN2 Deficient Macrophages Causes Up-Regulation of CHOP, GRP78, Caspase-3 and BAX

The interfering cell model was infiltrated with *B. abortus* A19, and the RNA and total protein of the infected cells were collected 24 h later. Results showed that the levels of mRNA transcript and protein expression of CHOP and GRP78, which are characteristic molecules of endoplasmic reticulum stress, in the interference group were significantly higher than those in the control group *(Fig. 3-A,B,C,D,G,H)*. The apoptotic molecules Cleaved caspase-3 and BAX mRNA transcript and protein expression levels were significantly higher in the interference group than in the control group *(Fig. 3-E,F,I,J)*. The results suggest that interference with the MFN2 gene enhances the ability of *B. abortus* A19 to induce endoplasmic reticulum stress and apoptosis.

B. abortus A19 Infection of Macrophages Overexpressing MFN2 Results in Down-Regulation of CHOP, GRP78, Caspase-3 and BAX

B. abortus A19 infection overexpression cell model, results showed that the endoplasmic reticulum stress signature molecules CHOP, GRP78 mRNA transcript level and protein expression level were significantly reduced in

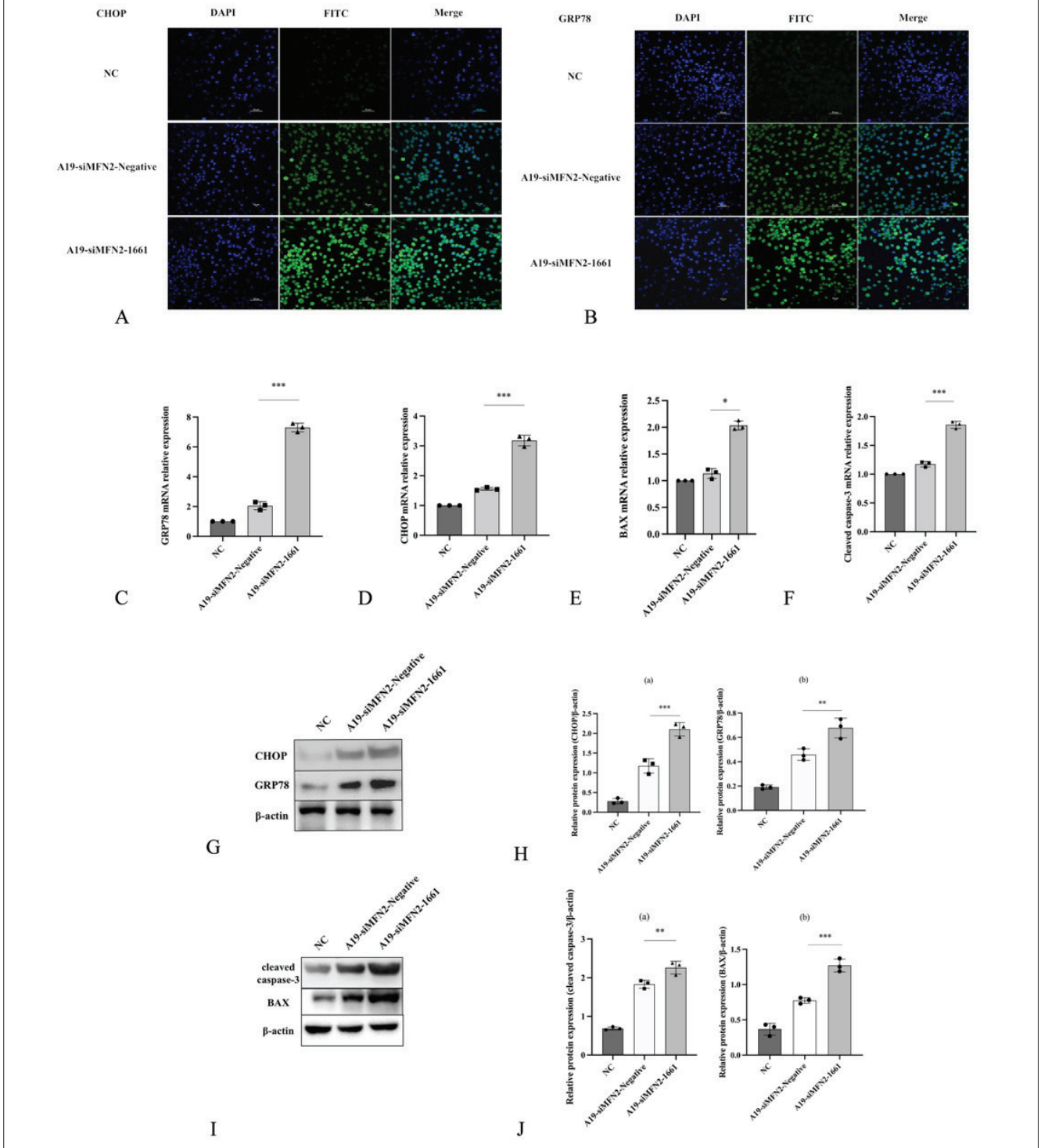

Fig 3. Expression levels of CHOP, GRP78, caspaes-3 and BAX were detected in the interfering cell model. (A) Confocal microscopy to detect CHOP spots 24 h after infection with the interfering cell model. CHOP spots are presented in green (FITC), while blue (DAPI) denotes nucleus, (B) Confocal microscopy to detect GRP78 spots 24 h after infection with the interfering cell model. GRP78 spots are presented in green (FITC), while blue (DAPI) denotes nucleus, (C)The expression level of GRP78 mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the interference cells model, (D) The expression level of CHOP mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the interference cells model, (E) The expression level of BAX mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the interference cells model, (F) The expression level of cleaved caspase-3 mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the interference cells model, (G) Expression levels of CHOP and GRP78 protein after infection with *B. abortus* A19 in the interference cells model, (H) The ratio of CHOP (a) and GRP78 (b) relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD, (I) Expression levels of Cleaved caspase-3 and BAX protein after infection with *B. abortus* A19 in the interference cells model, (J) The ratio of cleaved caspase-3 (a) and BAX (b) relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD. **P<0.05; **P<0.01; ***P<0.001*

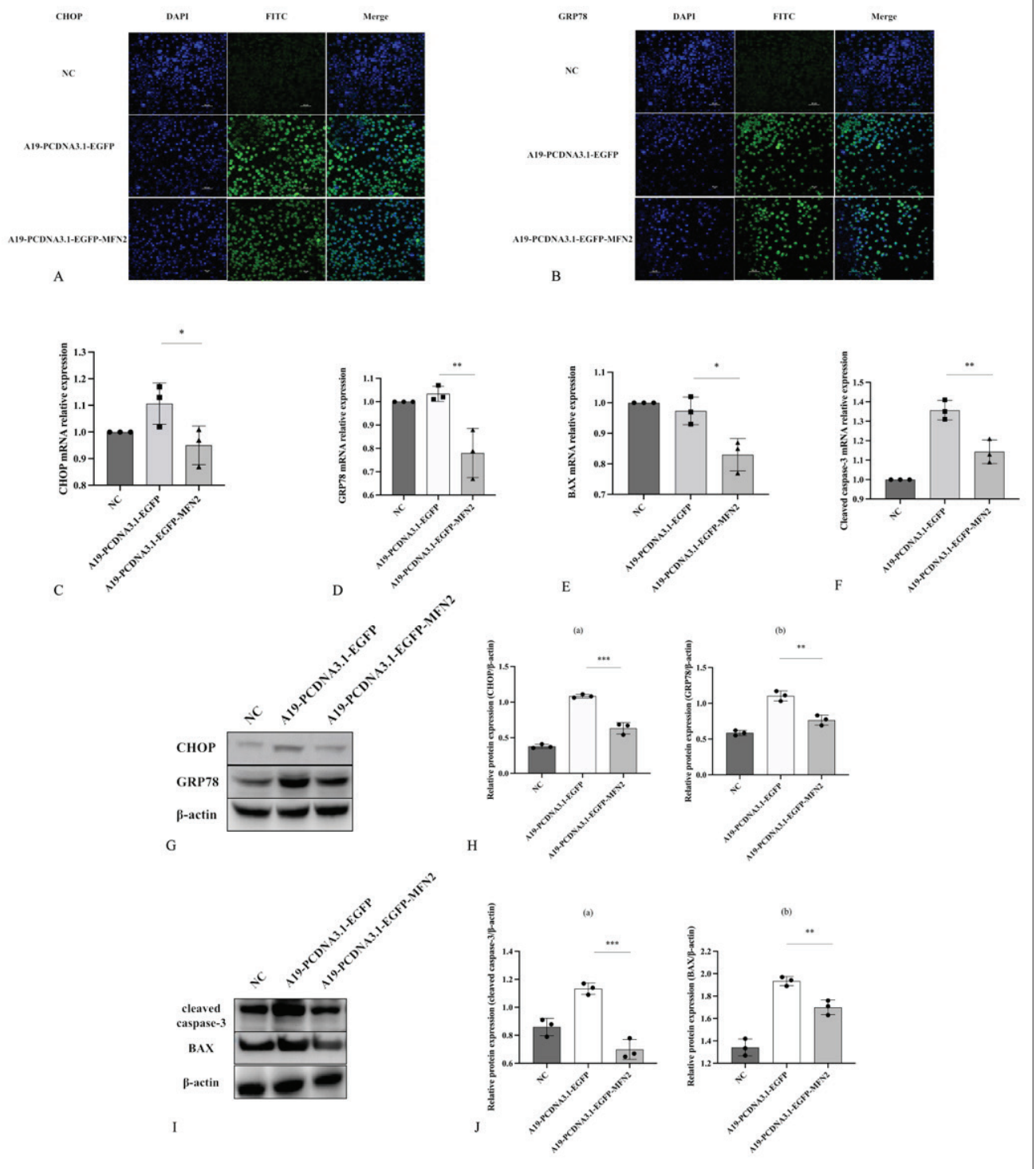

Fig 4. Expression levels of CHOP, GRP78, caspase-3 and BAX were detected in the overexpressing cell model. (A) Confocal microscopy to detect CHOP spots 24 h after infection with the overexpressing cell model. CHOP spots are presented in green (FITC), while blue (DAPI) denotes nucleus, (B) Confocal microscopy to detect GRP78 spots 24 h after infection with the overexpressing cell model, (C) The expression level of CHOP mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the overexpressing cell model, (D)The expression level of GRP78 mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the overexpressing cell model, (E) The expression level of BAX mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the overexpressing cell model, (F) The expression level of cleaved caspase-3 mRNA was detected by qRT-PCR after infection with *B. abortus* A19 in the overexpressing cell model, (G) Expression levels of CHOP and GRP78 protein after infection with *B. abortus* A19 in the overexpressing cell model, (H) The ratio of CHOP (a) and GRP78 (b) relative to β-actin levels was calculated using ImageJ. The experiment was repeated three times and data represent mean ± SD, (I) Expression levels of Cleaved caspase-3 and BAX protein after infection with *B. abortus* A19 in overexpressing cell model, (J) The ratio of cleaved caspase-3 (a) and BAX (b) relative to β-actin levels was calculated using ImageJ. The *experiment was repeated three times and data represent mean ± SD. *P<0.05; **P<0.01; ***P<0.001*

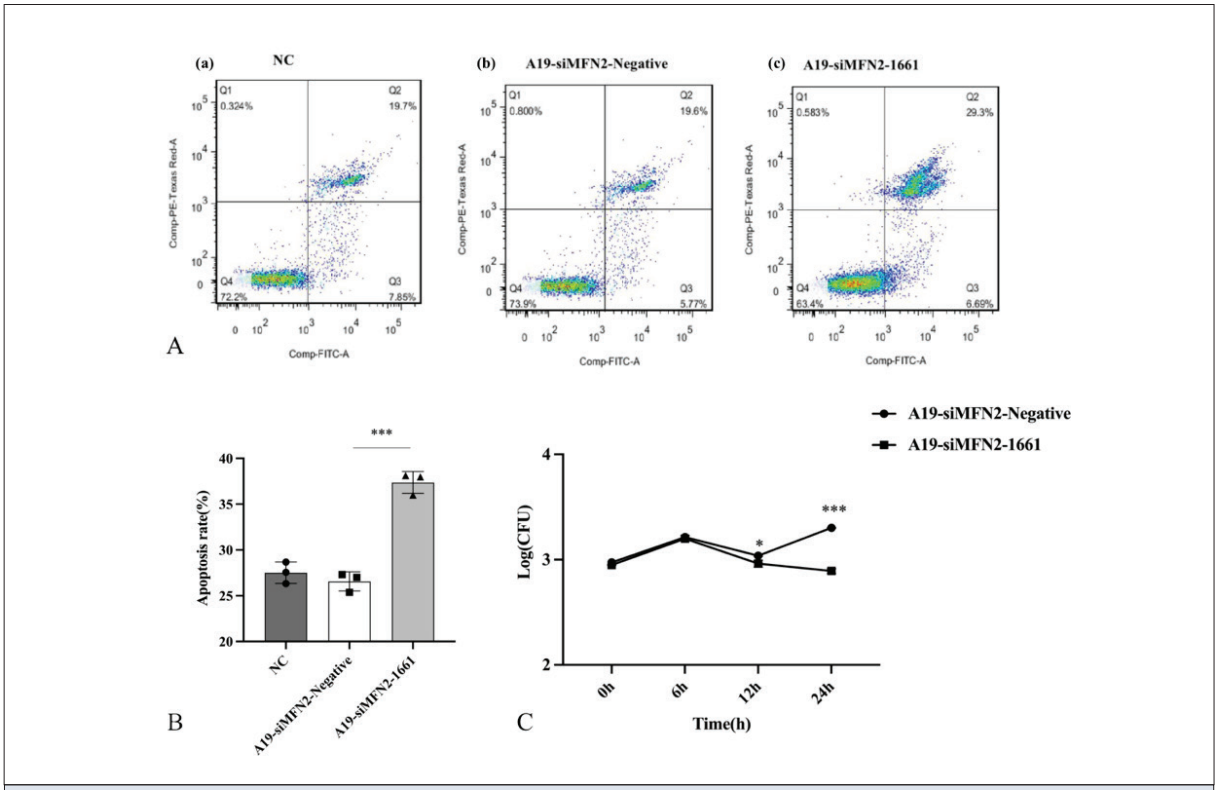

Fig 5. Apoptosis and *B. abortus* A19 intracellular survival in the *B. abortus* A19-induced interfering cell model. (A) Detection of apoptosis in *B. abortus* A19-induced interfering cell model cells by flow cytometry, (B) Bar graph of apoptosis rates in *B. abortus* A19-induced interfering cell models, (C) B. abortus A19 bacterial load in a model of interfering cells. ***P<0.001

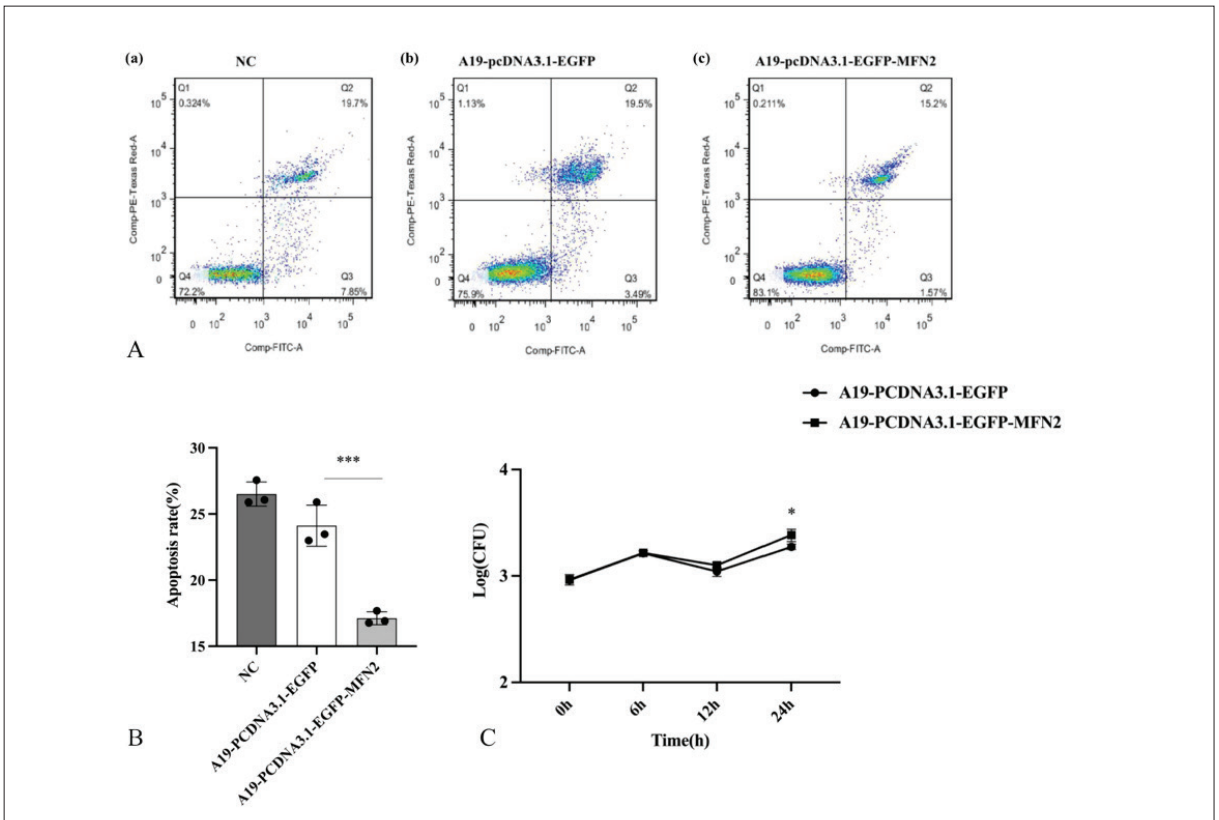

Fig 6. Apoptosis and *B. abortus* A19 intracellular survival in the *B. abortus* A19-induced overexpression cell model. (A) Detection of apoptosis in *B. abortus* A19-induced overexpression cell model by flow cytometry, (B) Bar graph of apoptosis rate in *B. abortus* A19-induced overexpressing cell models, (C) *B. abortus* A19 bacterial load in a model of overexpression cells. *P<0.05; ***P<0.001

the overexpression group compared with the control group *(Fig. 4-A,B,C,D,G,H)*, and the apoptosis signature molecules Cleaved caspase-3, BAX mRNA transcript and protein expression levels were also significantly reduced *(Fig. 4-E,F,H,J)*. This indicates that overexpression of MFN2 gene decreases the ability of *B. abortus* A19 to induce endoplasmic reticulum stress and apoptosis.

B. abortus A19 infection with Macrophages Lacking MFN2 Results in Decreased *B. abortus* A19 Intracellular Survival

B. abortus A19 infection was observed to have an impact on the MFN2 model cells. The cells were lysed at different time intervals and the number of viable bacteria inside the cells was measured. The results showed a significant decrease in the number of intracellular *B. abortus* A19 in the MFN2-interfered group at 12 h and 24 h, as compared to the control group (siMFN2-Negative group) (P<0.001) *(Fig. 5-C)*. Additionally, at 24 h post-infection, cells were collected and apoptosis was evaluated using flow cytometry. The findings indicated a significantly higher rate of apoptosis in the MFN2-interfered group, as compared to the control group (siMFN2-Negative group) (P<0.001) *(Fig. 5-A,B)*.

Infection of *B. abortus* A19 with Macrophages Overexpressing MFN2 Leads to an Increase in *B. abortus* A19 Intracellular Survival

B. abortus A19 was infected with cells that overexpressed the MFN2 model. The cells were then lysed, and the number of surviving bacteria within the cells was counted. The results showed a significant increase in the number of *B. abortus* A19 within the cells in the MFN2 overexpression group at 24 h, compared to the control group (PCDNA3.1-EGFP) (P=0.020) *(Fig. 6-C)*. Furthermore, at 24 h post-infection, cells were collected and assessed for apoptosis using flow cytometry. The results indicated a significantly lower apoptosis rate in the MFN2 overexpression group, compared to the control group (PCDNA3.1-EGFP) (P<0.001) *(Fig. 6-A,B)*. These results suggest that MFN2 plays a role in down-regulating programmed death in *B. abortus* A19-infected macrophages and supports the intracellular survival of *Brucella*.

Discussion

Brucella is a very "cunning" pathogen that evades the host immune system by translocating to the endoplasmic reticulum in the form of *Brucella* vesicles, leading to chronic infections [21,22]. Macrophages express high levels of MFN2, which not only regulates endoplasmic reticulum stress but also plays a crucial role in immune response regulation [23,24]. MFN2 is also involved in maintaining cellular autophagy. In the absence of MFN2 protein, autophagosomes accumulate, leading to reduced autophagic flux and increased apoptosis. However, this also results in attenuated apoptotic bodies and bacterial phagocytosis [25]. Pathogens have developed various mechanisms to manipulate MFN2 and influence their survival within host cells. In this study, we discovered that MFN2 is responsible for down-regulating endoplasmic reticulum stress and programmed cell death in *B. abortus* A19-infected macrophages, thereby supporting the intracellular survival of *B. abortus* A19.

Macrophages are the main target cells of *Brucella* infections, and in general, *Brucella* undergo immune escape by inhibiting apoptosis to ensure their intracellular survival [26], whereas macrophages themselves enhance the body's killing of pathogenic bacteria and control their intracellular multiplication by regulating apoptosis [27]. Among several apoptotic pathways, the endoplasmic reticulum stress-induced apoptosis pathway is favoured by scholars. The endoplasmic reticulum is involved in the maintenance of cellular homeostasis and can save calcium ions in large quantities, and it is also the main site of *Brucella* proliferation, when pathogenic bacteria infection, calcium ion homeostasis imbalance and redox environment disorders, it will lead to endoplasmic reticulum stress [28], if the endoplasmic reticulum homeostasis can not be restored for a long time, apoptosis will occur, and the ability of *Brucella* to induce cellular endoplasmic reticulum stress has been proved by a large number of scholars [29], the present study found that MFN2 disruption promotes *B. abortus* A19-induced expression of the endoplasmic reticulum stress signature molecule CHOP and GRP78, whereas MFN2 overexpression does the opposite, indicating that deletion of MFN2 enhances the ability of *B. abortus* to induce endoplasmic reticulum stress, which is similar to that found in the mouse fibroblast cell model of MFN2 gene deletion [23], which may be attributed to the fact that MFN2 deletion affected the coupling between the endoplasmic reticulum and mitochondria, disrupting the normal morphology of the endoplasmic reticulum, and possibly because MFN2 deletion interfered with the calcium transfer between the two, resulting in an imbalance of calcium ion homeostasis in the endoplasmic reticulum and aggravating the endoplasmic reticulum stress; in addition, we found that MFN2 interference enhanced the expression of Caspase-3 and BAX, a pro-apoptotic protein induced by *B. abortus* A19, and the flow results also further demonstrated that apoptosis rate increased after MFN2 interference, while the opposite was true for MFN2 overexpression, suggesting that MFN2 deficiency enhances *B. abortus*-induced apoptosis, which is similar to the findings of Lee et al. in *M. tuberculosis*-infected macrophages [30], which may be attributed to the fact that MFN2 deficiency interferes with the normal transmission of calcium ions between the endoplasmic

reticulum and mitochondria, resulting in mitochondrial calcium ions overload and apoptosis, or it may be because MFN2 deficiency exacerbates mitochondrial fragmentation and impairs mitochondrial function and apoptosis occurs, but these are only speculations, and the specific mechanisms remain to be investigated. The results of the intracellular survival assay showed that MFN2 interference decreased the viable bacterial count of *B. abortus* A19 in macrophages, while the opposite was true for MFN2 overexpression, suggesting that the ability of *B. abortus* A19 to survive intracellularly was suppressed to a certain extent after MFN2 deletion, which, combined with the results of the apoptosis assay, suggests that it may be due to the fact that MFN2 deletion exacerbated the cellular apoptosis, so that *Brucella* was deprived of a place to shelter its survival, and thus was recognised and removed by the host, this result is similar to the findings of Lee et al.[30] in *M. tuberculosis*-infected macrophages, and in contrast to the findings of Lobet et al.[31] who found that MFN2 deletion did not affect the survival of *Brucella* in Hela cells, and that the discrepancy the reason may be because RAW264.7 cells are professional phagocytes, which are more sensitive to *Brucella* invasion and have a stronger ability to bind to *Brucella* as well as phagocytose and clear *Brucella* compared to non-professional phagocytes, Hela, or it may be because the *B. abortus* A19 belongs to a weakly virulent strain, which is inherently weaker in terms of surviving in macrophages [32].

In conclusion, as a key regulator of the innate immune response during pathogenic infection, Mfn2 can mediate the down-regulation of endoplasmic reticulum stress and programmed death in *B. abortus* A19-infected macrophages and support the intracellular survival of *Brucella*. The present study provides experimental This study provides an experimental basis for a better understanding of the function of MFN2 gene during *Brucella* infection and the elucidation of the pathogenic mechanism of *Brucella*, as well as a basis for the development of potential targets for preventive and therapeutic programmes against brucellosis.

Declarations

Availability of Data and Materials: Not applicable.

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Research Article

Evaluation of Hematological, Proinflammatory and Tumor Markers in Bovine Cutaneous Papillomatosis

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Abstract

Cutaneous papillomatosis occurs as benign tumoral skin lesions in cattle. Although this condition is most commonly observed in young animals, it can occur in cattle of any age. Although tumor necrosis factor alpha, high mobility group box-1 protein, myxovirus resistance protein-1 have been investigated in various tumoral conditions in human medicine, the relationship between these markers and other hematological indices have not been investigated in bovine papillomatosis with benign tumoral lesions. The purpose of the study is to determine the levels of these markers and their relationship with each other and with hematological indices. The study comprised two groups: the bovine papillomatosis (BP) group, consisting of animals with papillomatosis, and the control group, consisting of healthy animals. Each group consisted of 10 animals of different breeds and genders with an age of 1-2 years. In the BP group, there were significant differences in total leukocyte count, neutrophil count, monocyte count, and neutrophil-lymphocyte ratio values, which were higher, and lymphocyte-monocyte ratio values, which were lower. The levels of high mobility group box-1 protein, myxovirus resistance protein-1, and tumor necrosis factor alpha were significantly higher in the BP group compared to the control group. In conclusion, this study showed that there were significant differences in hematological, inflammation and tumor markers in cutaneous papillomatosis.

Keywords: Bovine haematological values, Cutaneous papillomatosis, High mobility group box-1, Myxovirus resistance protein-1, Tumor necrosis factor alpha

Introduction

Papillomatosis in cattle typically manifests as benign tumoral structures in the cutaneous and mucosal epithelium. Lesions can occur at any age, although the onset of papillomatosis in cattle is usually around 2 years of age [1,2]. The literature now lists 14 BPV pathotypes, however there might be as many as 20 [3]. The disease spreads through direct contact, contaminated equipment and food, and the use of contaminated syringes. Additionally, inheritance, sunlight, nutritional and hormonal disorders, and immunosuppression are factors that play a role in the pathogenesis of the disease [4]. Arthropods have also been reported to play an important role in transmission. Skin papillomas can spread throughout the body and to the alimentary tract, mammary glands, genital mucosa, and lungs, causing respiratory difficulties, decreased milk yield, and weight loss in animals [5,6]. Inflammation in the tumor area is generally initiated locally by inflammatory proteins, cytokines and immune mediators. These tumor-associated mediators and cytokines are subsequently released into the bloodstream, activating other inflammatory mediators in circulation [7]. In human medicine, the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) are commonly used as prognostic and survival markers in various types of malignant cancers [8,9].

High mobility group box-1 protein (HMGB1) is released from necrotic and inflammatory cells [10]. Elevated levels of HMGB1 have been identified as a marker of cancer. HMGB1 diffuses from tumor cells along with other intracellular molecules [11]. Overaccumulation of HMGB1 has been reported in cervical carcinomas [12]. In addition, HMGB1 increases the stimulation of tumor necrosis factor alpha (TNF-α), interleukin 1 beta and other inflammatory cells, and has many functions such as activation of the innate immune system and dentritic cell activation in cancer [10].

TNF-α is a cytokine that is released as an indicator

of the activation of macrophages and other pro-inflammatory cytokines and is detected in the early stages of inflammation. Additionally, TNF-α plays a crucial role in regulating the immune system [13]. In viral infections, TNF-α exhibits antiviral properties by inhibiting virus replication [14]. TNF-α has been shown to effectively initiate inflammation and plays an active role in repair and regeneration processes following epidermal injury and infection [15].

Myxovirus resistance 1 (Mx1) gene appears to increase in vertebrate viral infections when stimulated by type I and III interferons (IFNs). Myxovirus resistance protein-1 (Mx1) serves as a valuable biomarker for IFN activity and the effectiveness of IFN treatment in specific cancer types [16]. It has been reported that Mx1 is associated with various human cancers. Although Mx1 has been implicated in antitumor activity, it is unclear how it affects immune cells in tumoral conditions [17]. Mx1 proteins are released upon stimulation of IFNs. Additionally, Mx1 proteins exhibit antiviral properties against many viruses, including influenza viruses [18].

The aim of this study is to determine whether markers such as NLR, LMR, TNF-α, HMGB1 and Mx1, which are used as tumor markers in human medicine, can also be used as tumor markers in bovine papillomatosis, a benign tumoral disease, and to investigate the levels of these tumor markers and their correlations with each other and with hematological index values such as NLR and LMR.

Material and Methods

Ethical Approval

The study was approved by Atatürk University Animal Experiments Local Ethics Committee (Decision no: 2023/13, Approval date: 20.11.2023). Before taking blood samples from the animals, an "Informed Consent Form" was obtained from the animal owners.

Animals

The animals used in the BP group consisted of animals brought to the Veterinary Hospital of Atatürk University, Faculty of Veterinary Medicine with complaints of skin lesions. The healthy group was comprised of animals of different breeds and genders, and animals with no pathological conditions in both clinical and hematological examinations were selected. Each group consisted of 10 animals of different breeds and genders.

Clinical and Hematological Examination

Routine clinical examination (examination of lymph nodes, heart and respiratory rate, body temperature, dehydration status, etc.), skin examination and hemogram analysis (Abacus junior Vet 5, Hungary) were performed on both healthy animals and animals with papillomatosis. The diagnosis of papillomatosis was made based on clinical observations. Animals with any other disease in their clinical and hematological examinations were excluded from the study. In order to create uniformity in the clinical examination of animals and to avoid different interpretations, an examination protocol was carried out by a researcher.

Blood Collection and Handling

Blood samples were taken from the jugular veins of the animals using a 20 mL needle (18 G, 1.20 x 38 mm, Berika, Türkiye). Hematological examinations of these blood samples were immediately performed by placing 2 mL into tubes containing ethylenediamine tetraacetic acid (EDTA) and their analyses were performed. For biochemical analyses, 8 mL blood samples were taken into serum tubes (BD Vacutainer® SST™ II Advance, UK) and kept at room temperature for 30 min. The serum tubes were then centrifuged in a refrigerated centrifuge (Beckman Coulter Allegra X-30R, USA) at 3000 rpm for 10 min. The sera obtained were transferred to Eppendorf tubes and stored

Table 1. *Comparison of mean ± SD and median values of hematological index in bovine papillomatosis and control group*

Parameters	**Control Mean±SD**	**BP Mean±SD**	**P**
WBC (×10³/μL)	7.72±1.19	18.76±3.78	<0.001
LYM (×10³/μL)	4.13±0.84	4.60±0.96	>0.05
NEU (×10³/μL)	3.34±0.95	13.22±3.06	<0.001
NLR	0.84±0.29	2.91±0.59	<0.001
LMR	23.51±4.07	6.12±2.01	<0.001
Parameter	**Median (Q1-Q3)**	**Median (Q1-Q3)**	**P/Mann Whitney U**
MON (×10³/μL)	0.15 (0.14-0.24)	0.74 (0.57-0.99)	<0.001/<0.0001

WBC: *Total leukocyte count;* ***LYM:*** *Lymphocyte;* ***MON:*** *Monocyte;* ***NEU:*** *Neutrophil;* ***NLR:*** *Neutrophil to lymphocyte ratio;* ***LMR:*** *Lymphocyte to monocyte ratio. P<0.05 is statistically significant*

Table 2. *Comparison of mean ± SD and median values of inflammation and tumor marker in bovine papillomatosis and control group*

Parameter	**Control Mean±SD**	**BP Mean±SD**	**P**
Mx1 (ng/mL)	8.66±2.59	12.83±3.00	<0.01
Parameters	**Median (Q1-Q3)**	**Median (Q1-Q3)**	**P/Mann Whitney U**
HMGB1(ng/mL)	26.82 (22.55-29.26)	46.42 (35.31-60.41)	<0.01/8.00
TNF-α (ng/L)	118.53 (114.19-132.43)	216.75 (195.36-243.69)	<0.001/<0.0001

BP: *Bovine papillamotosis;* ***Mx1:*** *Myxovirus resistance protein-1;* ***HMGB1:*** *High-mobility group box-1 protein;* ***TNF-α:*** *Tumor necrosis factor alpha. P<0.05 is statistically significant*

Table 3. Spearman correlation relations between values

Parameters	WBC	LYM	MON	NEU	NLR	LMR	HMGB1	Mx1	TNF-α
WBC	1.000	0.519*	0.916**	0.944**	0.864**	-0.842**	0.484*	0.634**	0.622**
LYM		1.000	0.542*	0.328	0.073	-0.292	-0.115	0.149	-0.011
MON			1.000	0.836**	0.728**	-0.928**	0.505*	0.576**	0.671**
NEU				1.000	0.946**	-0.816**	0.582**	0.652**	0.658**
NLR					1.000	-0.764**	0.652**	0.649**	0.711**
LMR						1.000	-0.610**	-0.597**	-0.762**
HMGB1							1.000	0.295	0.713**
Mx1								1.000	0.701**
TNF-α									1.000

WBC: *Total leukocyte count;* ***LYM:*** *Lymphocyte;* ***MON:*** *Monocyte;* ***NEU:*** *Neutrophil;* ***NLR:*** *Neutrophil to lymphocyte ratio;* ***LMR:*** *Lymphocyte to monocyte ratio;* ***HMGB1:*** *High-mobility group box 1 protein;* ***Mx1:*** *Myxovirus resistance protein-1;* ***TNF-α:*** *Tumor necrosis factor alpha. * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level*

in a deep-freeze refrigerator (Esco Lexicon® ULT Freezer, USA) at -80°C until analysis.

Assays for Serum High Mobility Group Box-1 Protein, Tumor Necrosis Factor Alpha, and Myxovirus Resistance Protein-1

Serum levels of HMGB1, TNF-α, and Mx1 were measured using a commercial ELISA test kit (Bovine BT Lab., Zhejiang, China) following the manufacturer's instructions. The ELISA procedures were also processed and shaped in the ELISA reader device (BioTek μQuant MQX200 ELISA Reader, USA).

Statistical Analysis

A study was conducted on cattle with papillomatosis to evaluate their peripheral blood hematologically and immunophenotypically. Each group consisted of 5 cattle. The study also included a power analysis of hematologic index values (≥95% power ratio (effect size=2.84; α=0.05; distribution ratio=1:1), which revealed that the study could be conducted on at least 4 animals [19]. Power analysis was performed using G*Power® version (3.1.9.4, Franz Faul, Universität Kiel, Germany). Data analysis was performed using IBM SPSS Statistics 27.0.1 software.

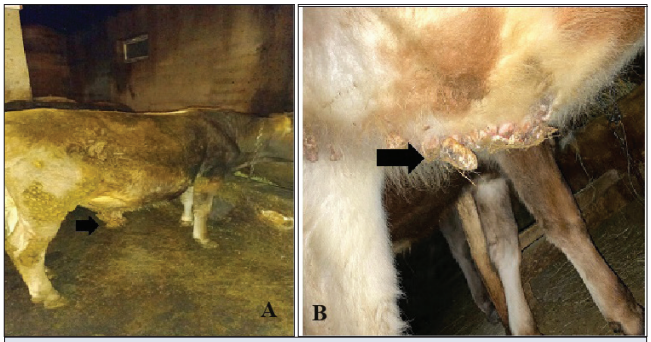

Fig 1. A-B: Umbilical and abdominal papillomatosis lesions. The arrow indicates the papillomatosis lesion

The Shapiro-Wilk normality test was used to assess the normal distribution of the data, and Levene's test was conducted to determine the homogeneity of the data. Descriptive statistics were presented as number of units (n) and mean ± standard deviation ($\bar{x}$±sd) for normally distributed data and as quartiles (Q1-Q3) for non-normally distributed data. To determine the difference between groups, the independent samples *t*-test was used for normally distributed data, and the Mann-Whitney U test was used for non-normally distributed data. Spearman correlation analysis was used to determine the correlation between the data. As stated by Chan [20], a correlation coefficient (*rho*) <0.3 is considered as a very weak correlation, 0.3-0.5 is considered as a moderate correlation, 0.6-0.8 is considered as a strong correlation, and a correlation coefficient value of ≥0.8 is considered as a very strong correlation in Spearman's correlation coefficient analysis.

Results

Clinical and Hematological Findings

In clinical examinations of cattle with papillomatosis, it was determined that cauliflower-like skin lesions were formed in the abdominal region and linea alba region *(Fig. 1)*. Although other clinical examinations (such as respiration rate, pulsation, rectal temperature) of these animals were within the reference range, hematological examinations revealed that the values of total leukocyte count (WBC), neutrophil count (NEU), monocyte count (MON) and NLR were significantly higher in the BP group than in the control group (P<0.001). Additionally, LMR value in the BP group was statistically lower than that in the control group (P<0.001). However, there was no significant difference between the groups in terms of lymphocyte count (LYM) (P>0.05) *(Table 1)* *(Fig. 2)*. Animals in both groups consist of 1-2 years old animals.

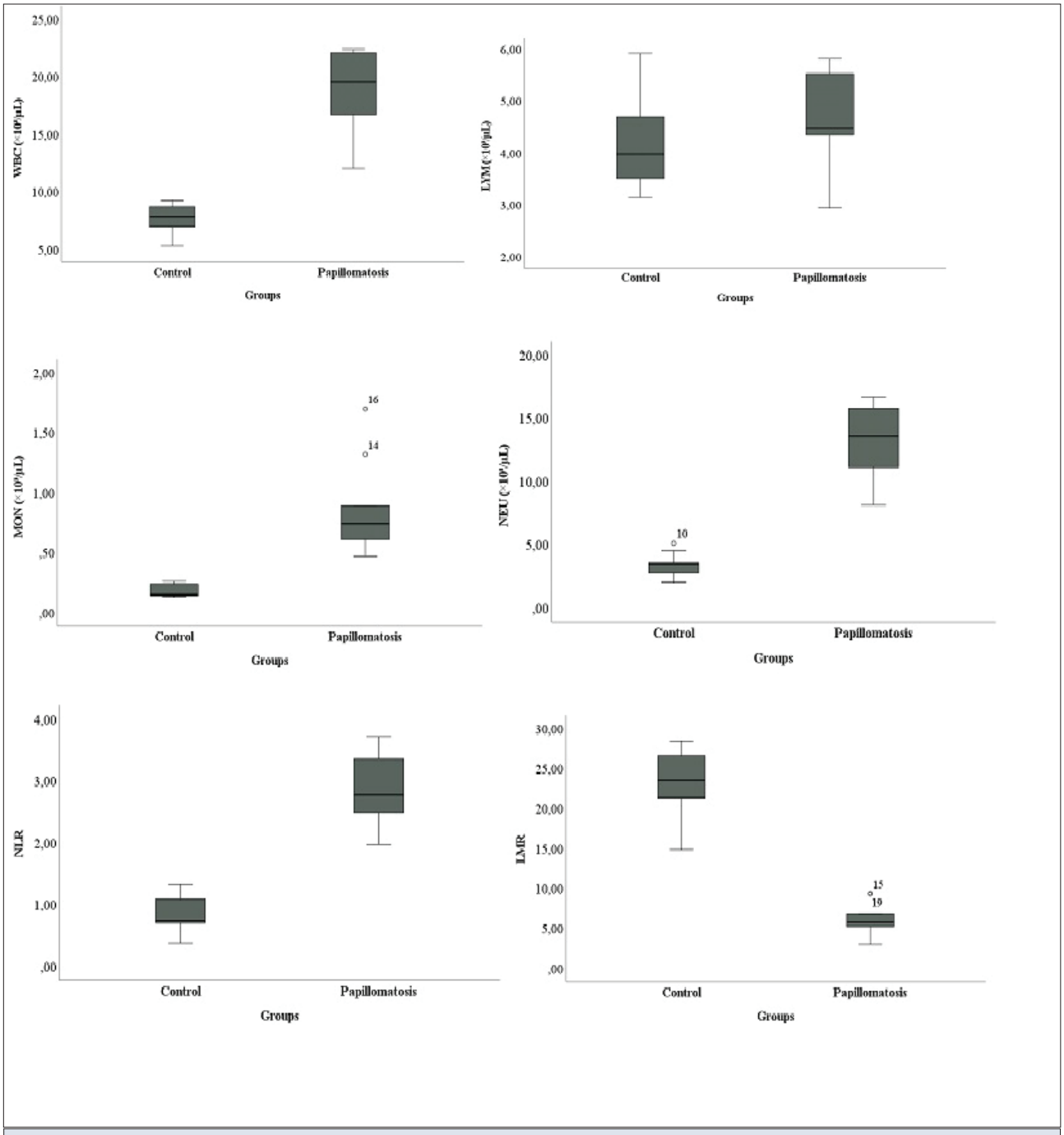

Fig 2. Boxplot diagram of hematological values between groups. Circles indicate outliers

Inflammation and Tumor Marker Findings

Table 2 shows the serum values of HMGB1, TNF-α, and Mx1. Cattle with BP had significantly higher serum Mx1, HMGB1 and TNF-α levels compared to the control group (P<0.01; P<0.01; P<0.001, respectively) *(Fig. 3)*.

Correlation Findings of Hematological, Inflammatory and Tumor Markers

The correlation table for these markers is shown in *Table 3*. WBC was moderately correlated with LYM and HMGB1 (*rho*=0.519; P<0.05, *rho*=0.484; P<0.05, respectively). WBC was found to be very strongly correlated with MON, NEU, NLR and LMR (*rho*=0.916, 0.944, 0.864, and -0.842; all P values<0.01, respectively). WBC showed a strong correlation with both Mx1 and TNF-α (*rho*=0.634; P<0.01; *rho*=0.622; P<0.01, respectively). There was a moderate correlation between the values of LYM and MON (*rho*=0.542; P<0.05). The MON value showed a very strong correlation with both NEU and LMR (*rho*=0.836, -0.928, respectively; both P values <0.01). The MON value showed a strong correlation with both NLR and TNF-α (*rho*=0.728; 0.671, respectively; both P values

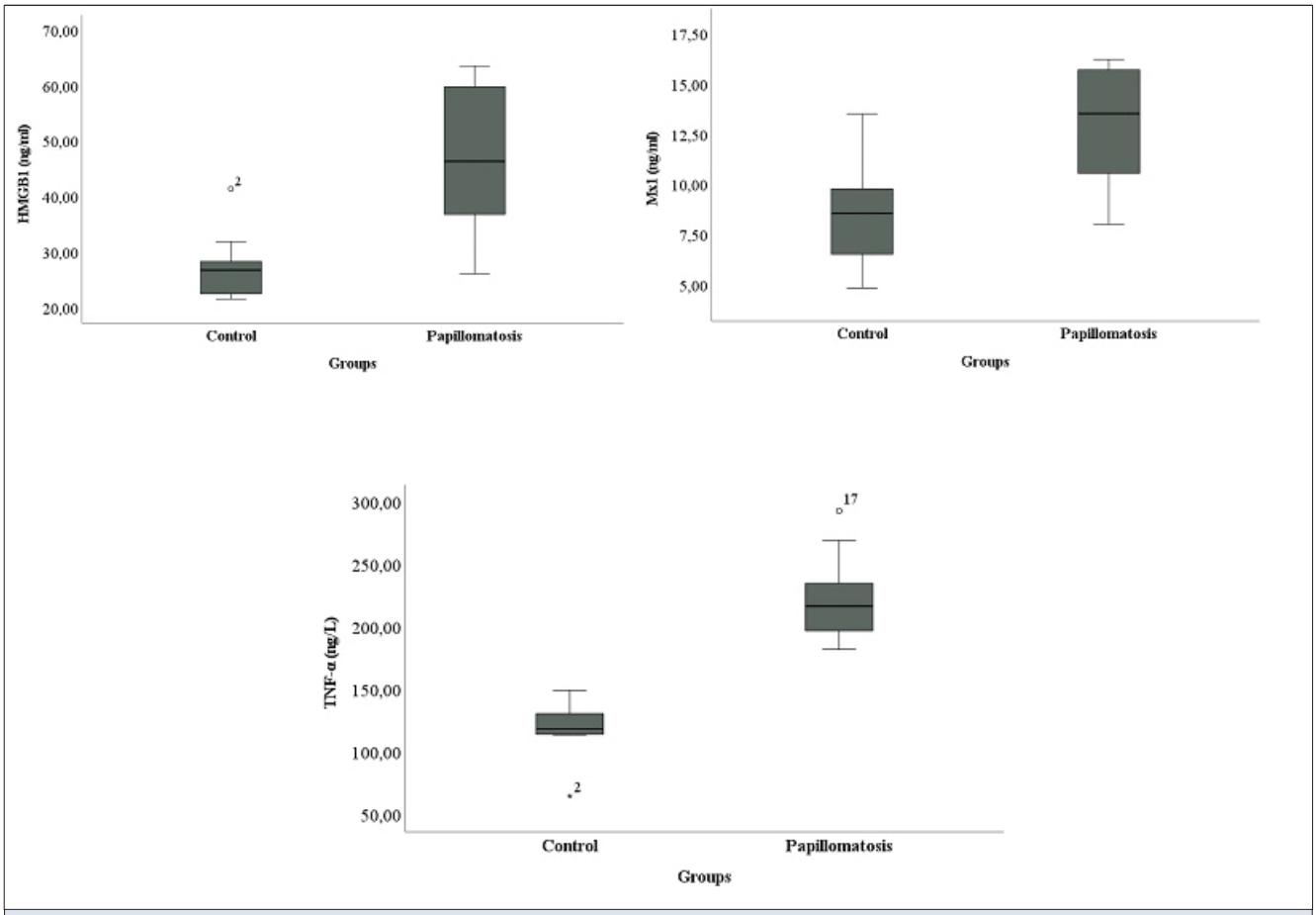

Fig 3. Boxplot diagram of inflammatory and tumor markers values between groups. Star and circles indicate outliers

P<0.01). The MON value showed moderate correlation with HMGB1 (*rho*=0.505; P<0.05) and Mx1 *(rho*=0.576; P<0.01). NEU was very strongly correlated with both NLR *(rho*=0.946, P<0.01) and LMR *(rho*=-0.816, P<0.01). A moderate correlation was found between NEU and HMGB1 *(rho*=0.582; P<0.01), while a strong correlation was found between NEU and Mx1 and TNF-α *(rho*=0.652, 0.658, respectively; both P values <0.01). NLR was found to be strongly correlated with LMR, HMGB1, Mx1, and TNF-α *(rho*=-0.764, 0.652, 0.649, 0.711, respectively; all P values <0.01). LMR was found to have a strong correlation with HMGB1 and TNF-α *(rho*=-0.610, -0.762; P<0.01, respectively). Additionally, LMR was moderately correlated with Mx1 *(rho*=-0.597; P<0.01). Strong correlations were obtained between HMGB1 and TNF-α *(rho*=0.713; P<0.01) and between Mx1 and TNF-α *(rho*=0.701; P<0.01). The correlation of the values is also presented in *Fig. 4.*

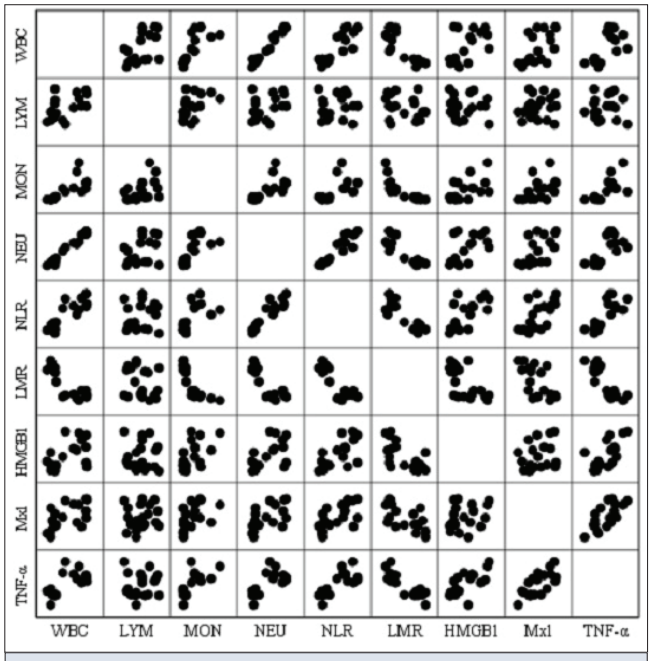

Fig 4. Correlation coefficients among hematolological index, inflamation and tumor markers in cattle with cutaneous papillomatosis. **WBC:** Total leukocyte count; **LYM:** Lymphocyte; **MON:** Monocyte; **NEU:** Neutrophil; **NLR:** Neutrophil to lymphocyte ratio; **LMR:** Lymphocyte to monocyte ratio; **HMGB1:** High mobility group box 1 protein; **Mx1:** Myxovirus resistance protein-1; **TNF-α:** Tumor necrosis factor alpha

Discussion

Bovine papillomatosis is a viral disease caused by bovine papillomaviruses that result in benign tumor-like skin lesions [21]. Skin papillomas in cattle mostly occur on the head and neck, and in some animals, on other parts of the

body [22]. In this study, cutaneous papillomatosis lesions were found on the linea alba and around the abdomen in all animals in the BP group. It was also observed that the age of the animals affected by cutaneous papillomatosis in the current study was between 1-2 years of age.

Imbalances in WBC have been shown to indicate a poor prognosis in hepatocellular carcinoma [23]. However, since there is a balance between neutrophil and lymphocyte counts in maintaining the immune system, polymorphonuclear leukocytes and WBC alone are not reliable prognostic markers [24]. On the other hand, NLR has been shown to be a prognostic marker in certain types of cancer [25]. Lymphocytes actively regulate the immune system in tumoral conditions. Therefore, low lymphocyte levels can trigger both tumor development and increased susceptibility to metastasis [26]. Circulating monocytes have been reported to trigger tumor cell growth and aid in their evasion of the immune system's control [27]. It has been reported that papillomavirus-like particle structures interact with immune system cells such as monocytes, dendritic cells, B lymphocytes, and this is shaped by the effect of the immune response to papillomavirus [28]. Additionally, there is a strong correlation between systemic inflammation and the development of cancer. While the antitumor effects of neutrophils are well-established, it has been reported that an increase in the relative number of neutrophils can also lead to an increase in inflammatory markers, such as anti-apoptotic substances and pro-angiogenic growth factors [29]. IL-6 and TNF-α are the major cytokines that increase the spreading of polymorphonuclear neutrophils [30]. A study on cattle with mastitis reported an increase in NLR levels parallel to the increase in TNF-α, caused by an increase in the inflammatory response [31]. Although the hematological examinations of healthy animals were within normal physiological limits, the study found a higher NLR rate in cattle with cutaneous papillomatosis and a lower LMR rate. It is seen that the reason for the change in NLR ratio here is due to the increase in inflammatory mediators [29,31], and the change in LMR status is due to monocytosis. The significant increase in monocyte levels in the BP group may be due to an immune system response to papillomavirus [28].

During the early phase of inflammation, TNF-α induces other chemokines that direct neutrophils and macrophages towards the skin epidermis. Furthermore, TNF-α contributes to the repair of damaged skin, in addition to initiating the innate immune system and inflammatory phase [32]. A study conducted in cattle with lumpy skin disease (LSD) reported that the increase in proinflammatory cytokine levels (IL-1β, IL-6, and TNF-α) may be caused by the systemic inflammatory response in LSD [33]. Another study conducted in cattle with trichophytosis found a significant increase in TNF-α levels in the patient group compared to the control group, possibly due to the increase in the inflammatory state [34]. In this study, like the previous studies, cattle in the BP group showed a significant increase in TNF-α levels compared to the control group. This may be due to the systemic inflammatory response, as noted by Kamr et al.[33]. The study found strong correlations between HMGB1 and TNF-α *(rho*=0.713; P<0.01), Mx1 and TNF-α *(rho*=0.701; P<0.01), and NLR and TNF-α *(rho*=0.711; P<0.01), suggesting the formation of an inflammatory response in cattle with cutaneous papillomatosis.

Mx1 is an indicator of the antiviral status induced by IFNs in many species. Studies have shown that Mx proteins inhibit the activity of Orthomyxoviridae, Rhabdoviridae, and Bunyaviridae viruses [35,36]. Additionally, it regulates the activity of IFNs in controlling normal and tumor cell metastasis. Mx1 has therefore been shown to be a tumor suppressor and a prognostic marker in tumoral conditions [37]. Evidence of a strong link between Mx1 protein and cutaneous squamous cell carcinomas has been reported due to marked hemostasis impairment caused by mutations (L95P, P96S, and P218S) [38]. A study conducted on patients with prostate cancer found high levels of Mx1. It was concluded that Mx1 plays an important role in suppressing tumor progression in this type of cancer [39]. Mx1 may be an indicator of disease resistance in livestock [40]. In this study, Mx1 levels were significantly higher in cattle in the BP group compared to the control group. It is thought that this may be related to both the ability of Mx1 to inhibit virus replication [36] and its suppressive property in tumoral tissues [39]. In cattle administered lipopolysaccharide (LPS) during early pregnancy, an increase in Mx1 gene expression was observed alongside an increase in TNF-α levels in response to an inflammatory stimulus. It has been suggested by the authors that this may be a regulatory response to the uterine proinflammatory response [41]. In the present study, a strong correlation was found between TNF-α and Mx1 *(rho*=0.701; P<0.01), suggesting that an increase in Mx1 status may serve as a regulatory response against the inflammatory state in cutaneous papillomatosis.

HMGB1 is a proinflammatory cytokine that is released by activated monocytes and dendritic cells or damaged and dead cells [42]. Extracellular HMGB1 has many important functions, such as inducing TNF-α, IL-1β, and other inflammatory cytokines, as well as promoting dendritic cell maturation. These processes play an active role in the chronic inflammatory process in cancer diseases [10]. In viral infections, such as respiratory syncytial virus [43], herpes simplex virus type-1 [44], and herpes simplex virus type-2 [45], HMGB1 levels have been reported to increase in direct proportion to the severity of the inflammatory

condition. Similarly, in a study conducted in cattle with bovine herpes virus-1, HMGB1 levels were shown to increase [46]. In this study, HMGB1 levels were found to be higher in cattle in the BP group compared to the control group, which may be related to the severity of the inflammatory condition, as stated by Workenhe et al.[45]. HMGB1 can activate nuclear factor kappa B (NF-κB), which in turn increases the stimulation of inflammatory cytokines such as TNF-α and IL-1β [47]. The strong correlation between HMGB1 and TNF-α in this study (*rho*=0.713; P<0.01) indicates the severity of the inflammatory state in cutaneous papillomatosis.

There are several limitations in this study. Although clinical findings can be used to diagnose cutaneous papillomatosis, other methods such as histopathology, electron microscopic examination, polymerase chain reaction, and viral genetic analysis can be used to determine the type of virus [48-50]. In the animals included in the study, diagnosis was made only on the basis of clinical findings of cutaneous lesions. Additionally, while the number of animals used in the study was supported by power analysis, it is possible that different results may be obtained in a study with a larger patient group. Finally, only a single blood sample was collected from both patient and control animals in this study. In addition to the markers investigated in the current study for this disease, studies conducted with different inflammatory markers, such as acute phase proteins, and simultaneous investigation of repeatedly taken blood samples may provide additional information about the disease.

In conclusion, this study found significant changes in hematological parameters, HMGB1, and Mx1, which are used as inflammatory and tumor markers, as well as TNF-α, a proinflammatory marker, in bovine cutaneous papillomatosis. These markers may provide useful information in determining the inflammatory status of the disease.

Declarations

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

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RESEARCH ARTICLE

Molecular Investigation of Viral Agents in Cattle with Respiratory System Problems#

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Abstract

This study was carried out to investigate viral etiology using molecular methods in cattle with respiratory system problems. The animal material of the study consisted of 200 cattle of different breeds, age and sex, which were not vaccinated against bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV) and Bovine adenovirus-3 (BAV-3), brought to the clinics of Kafkas University Faculty of Veterinary Medicine, Department of Internal Medicine, with respiratory system complaints and found to have abnormal respiratory system findings after clinical examination. Blood and swab (if animals had nasal discharge) samples were collected. PCR was performed, positive samples were sent for sequence analysis and the data were subjected to phylogenetic analysis. BVD was detected in 3 of the blood samples collected. From the swab samples; BVD was detected in 8 animals, BPIV-3 in 3 animals, and BRSV in 4 animals. BAV-3 was not detected in any of the collected samples. As a result of the phylogenetic analysis, it was determined that BVD viruses were classified as BVDV-1a, BPIV-3 viruses as genotype C and BRSV viruses as subgroup III (subgroup A). It is thought that these data will be help in guiding in the struggle against viral agents that frequently cause respiratory system diseases.

Keywords: Cattle, Respiratory system, BPIV-3, BVDV, BRSV, BAV-3

Introduction

The etiology of respiratory system diseases in cattle has a complex structure. This disease can be caused by bacteria and viruses and is called Bovine respiratory disease complex [1-3]. The diagnosis of this disease complex is usually made by multiplex ELISA kits [4,5]. Viral agents frequently involved in this disease complex generally include bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV) and bovine adenovirus (BAV) [4,6].

Bovine adenovirus (BAV) is a non-enveloped DNA virus and belongs to the *Adenoviridae* family. BAV type-3 is found within *Mastedenovirus* genus [7,8]. The agent, which has ten serotypes (BAV 1-10), plays a role in respiratory and enteric infections causing clinical findings such as conjunctivitis, pneumonia, polyarthritis, and diarrhea in calves [9].

BRSV is also known as Bovine orthopneumovirus. Virus is classified in genus *Pneumovirus* which belongs to the *Pneumoviridae* family. Its genetic material is a single-stranded enveloped RNA. The most severe clinical signs of Bovine respiratory syncytial virus (BRSV) are observed in calves aged 2 weeks to 6 months [10-12]. BRSV can be asymptomatic or cause respiratory infections in varying severities. Although cattle under six months of age are more susceptible to the disease, it can be seen frequently in cattle aged 3-12 months [13].

Bovine Parainfluenza type 3 (BPIV-3) is located in the *Respirovirus* genus in the *Paramyxoviridae* family [14-16]. The causative agent is an enveloped RNA virus. It causes clinical outcomes ranging from asymptomatic infections to serious respiratory problems. Cough, fever and nasal discharge are the main clinical findings [17].

Bovine Viral Diarrhea (BVD) virus which is classified

in the genus *Pestivirus* and *Flaviviridae* family [18,19], has an RNA genome [20]. It can cause abortion, diarrhea, respiratory system symptoms or severe pneumonia by involving in mix infections with other agents [18].

The aim of this study was to determine the presence of viral agents in cattle with respiratory system problems and make molecular characterization of these strains.

MATERIAL AND METHODS

Ethical Approval

This study is approved by Kafkas University Local Ethics Committee of Animal Experiments with permission number (KAÜ-HADYEK/2020-031).

Sampled Animals

The study included 200 cattle of different age, sex and breed with clinical respiratory system symptoms that were brought to the clinics of the Department of Internal Medicine, Faculty of Veterinary Medicine, Kafkas University. These animals were not vaccinated against the viruses that were investigated in the study (BPIV-3, BVDV, RSV and BAV-3). From these cattle, 200 blood and 63 nasal swab samples were collected. Swab samples were collected from animals with nasal discharge.

Blood Samples

Blood was collected from the *vena jugularis* into tubes with EDTA (BD Vacutainer®, BD, UK). Blood samples in EDTA tubes were centrifuged at 2000 rpm for 10 minutes. The leukocyte layer was removed with a Pasteur pipette and transferred to stock tubes containing 2 mL Phosphate Buffer Saline (PBS). The samples were stored in a -20°C deep freezer until testing.

Nasal Swab Samples

The swabs were taken by pressing and rubbing to the nasal cavity. Samples were delivered to the laboratory in cold chain. The sticks of the swabs were discarded, tips were put in stock tubes with 2 mL PBS and vortexed vigorously. Afterwards, they were centrifuged in a refrigerated centrifuge at 3000 rpm for 10 min at +4°C. The supernatant was transferred to clean stock tubes and stored in -20°C deep freezer for PCR applications.

DNA/RNA Extraction

The method described by Sambrook et al.[21] was used to extract both DNA and RNA. Extracts of the samples were stored at -20°C until molecular analyses.

Molecular Procedures

After extraction of viral nucleic acids from the samples, complementary DNA synthesis and PCR for BPIV-3, BRSV, BVDV viruses was achieved with 2x One-step RT-PCR master mix (Hibrigen) enzyme kit according to the manufacturer's instructions. For BAV-3, Taq polymerase kit (Hibrigen, Türkiye) was used for PCR after extraction. Primer pairs and optimizations for PCR were applied according to references [9,17,22,23].

Sequencing and Phylogenetical Analyses

Positive samples obtained after PCR/RT-PCR procedures were selected and sent for sequence analysis to a commercial company (BM Lab, Ankara, Türkiye). Raw data from sequence analysis were processed and phylogenetic trees were constructed for each virus. Sequence alignment was performed with Bioedit (Version 7.0.5.3) [24]. Sequence similarities were compared by using the GenBank database and BLAST software (NCBI) [25]. Phylogenetic analysis of gene sequences was performed using MEGA7 software [26]. Neighbor-joining method was chosen for comparison and sequence divergence was calculated by Kimura two-parameter model; confidence level was assessed by bootstrapping using 1000 replicates.

RESULTS

A total of 200 animals were included in the study. The ages of the animals were ranged from 1 day to 7 years old. The age distribution of the animals included in the study is shown in *Table 1*. Of these animals, 84 were female and 116 were male. The breed distribution was 178 Simmental, 3 Holstein hybrids, 5 local breed, 1 Zavot, 12 Brown Swiss and 1 hybrid breed. It was noteworthy that the majority of the animals included in the study were of Simmental breed. All animals had clinical respiratory symptoms (nasal discharge, cough, dyspnea etc.)

It was observed that respiratory system infections were more common in animals up to 1 year of age than in older animals.

A total of 200 blood and 63 swab samples were collected from 200 animals. While three of the blood samples were positive for BVDV, no positivity for other viral agents was detected in the blood. In comparison, BVDV was detected in eight samples, BPIV-3 in three samples and BRSV was detected in four samples from the collected swabs. While three blood samples were positive for BVDV, swab samples of the same animals were also positive. Consequently, eight animals were found to be positive for BVDV.

Table 1. *Age distribution of the animals included in the study*

Age	Number	Ratio (%)
1 day-1 month	54	27%
>1month-≤3 months	49	24.5%
>3months-≤1 year	55	27.5%
>1 year-≤3 years	19	9.5%
>3 years	23	11.5%

Given the unavailability of a positive control, BAV-3 was analyzed without the benefit of such a control. The results demonstrated that BAV-3 was not detected in any of the collected samples.

Of the animals positive for viral agents, 13 were Simmental (7 BVDV, 3 BPIV-3, 3 BRSV), 1 was Brown Swiss (BRSV) and 1 was local breed (BVDV).

It was noted that 6 of the BVD positive animals were 4-7 months old, 1 was 18 months old and 1 was 2 years old; BPIV-3 positive animals were 15 days, 3 months and 4 months old; and 4 BRSV positive animals were 2-7 months old.

It was observed that 13 of the 15 animals positive for viral agents were male and 2 were female.

No mixed infection with respect to viral agents was found in any of the positive animals. Respiratory distress, nasal discharge, coughing, and anorexia were observed in all positive animals.

Table 2. *Vital signs of the virus positive animals*

Viral Agent	Mean Rectal Temperature (°C)	Mean Pulse Rate (Frequency/min)	Mean Respiratory Rate (Frequency/min)
BVDV (swab) (n=8)	39.45	98	42.5
BPIV-3 (n=3)	39.8	82.67	50.67
BRSV (n=4)	39.35	111.5	44

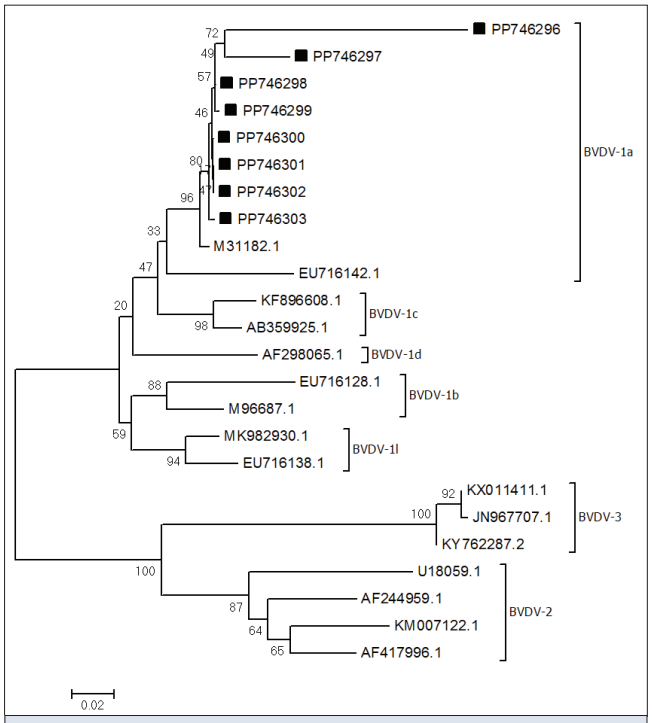

Fig 1. Phyogenetic tree constructed for BVDV using study's sequence data. Study isolates are shown with a black squares

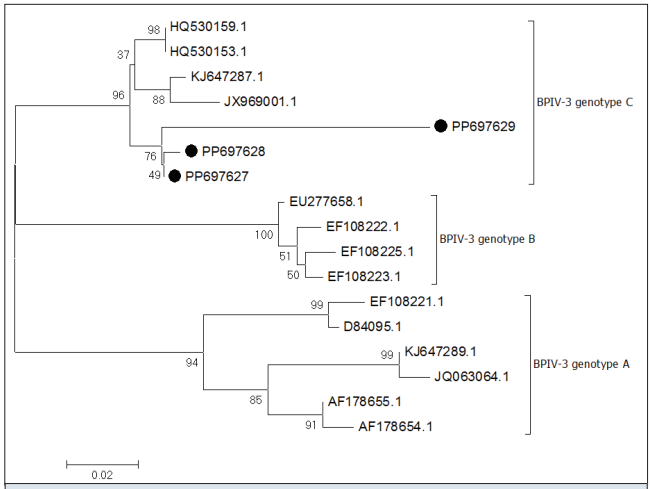

Fig 2. Phyogenetic tree constructed for BPIV-3 using study's sequence data. Study isolates are shown with a black dots

The mean rectal temperature, pulse rate and respiratory frequency of all animals included in the study (n=200) were 39.02°C, 101.38/min and 43.07/min, respectively. The mean rectal temperature, pulse rate and respiratory frequency of the animals (n=185), which were included in the study but had no virus infection, were 38.98°C, 101.61/min and 42.95/min, respectively. The vital signs of the positive animals are shown in *Table 2*.

It was found significant that 6 of the animal included in the study belonged to the same owner and 3 swab samples belonging to these animals were tested positive for BVDV.

Positive amplicons were subjected to sequence analysis and obtained sequences were submitted to GenBank for archiving. The accession numbers of three isolates of BPIV-3 were PP697627, PP697628, PP697629; four isolates of BRSV were PP719911, PP718912, PP718913, PP718914; eight isolates of BVDV were PP746296, PP746297, PP746298, PP746299, PP746300, PP746301, PP746302, PP746303. Given that the sequence data obtained from the blood and swab samples of the same animals were identical, no accession number was assigned to the sequence derived from the blood samples.

As a result of the phylogenetic analyses, the BVDV isolates found in the study were classified as BVDV-1a *(Fig. 1)*, BPIV-3 isolates were classified as genotype C *(Fig. 2)* and BRSV isolates were classified as subgroup III (subgroup A) *(Fig. 3)*. It was determined that the viruses found were similar to the strains previously encountered in Türkiye.

DISCUSSION

Respiratory diseases can cause severe economic losses in sheep [27] and cattle [28]. Among these, viral agents have an important place in cattle and are widely observed in Türkiye [28]. In addition to the disease symptoms they

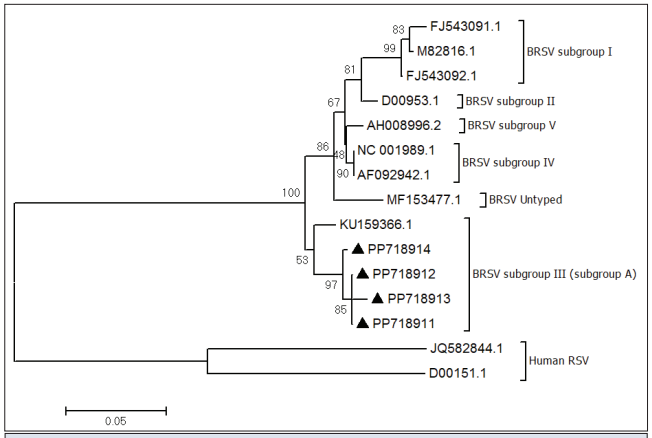

Fig 3. Phyogenetic tree constructed for BRSV using study's sequence data. Study isolates are shown with a black triangles

cause, viral agents that cause respiratory system infections can also suppress the immune system by altering immune mechanisms [29-31].

The success of vaccination against diseases is affected by environmental factors such as proper vaccination procedures and herd management [32]. In addition, the protection of vaccines may change due to the emerging of new strains with ongoing mutations in viruses [33,34]. Phylogenetic reports obtained from regional studies are important for the evaluation of vaccine efficacy. For this reason, it is an important strategy in the fight against diseases to continuously identify and reveal field strains by competent laboratories and to revise vaccine preparations according to this data if different strains are found [32]. The phylogenetic analysis results obtained from this study contributed to the literature in this regard.

One of the problems that occur in multiple infections, since it can be caused by many microbial agents (such as bacteria and viruses) [4], the role of some agents in the clinical picture may be overlooked. Even if one of the agents in infection has a minor role, prognosis may be worsened if that agent is not diagnosed and treated accordingly. Mixed viral infection was not found in this study, this situation has been reported in seroprevalence studies [4,35-38]. Although seroprevalence values vary in studies [36,37], it is seen that viruses infecting the respiratory system frequently coexist. As vaccines against respiratory viruses are usually in combined form, they offer protection against more than one disease. In this regard this situation seems to be advantageous for current picture of the field.

Serologic presence of BVDV has been known for a long time, and its contribution to respiratory system problems has been reported by previous studies, according to these data, the virus is widespread in Türkiye [30,39,40]. Molecularly, BVDV is classified as BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B) and BVDV-3 (Pestivirus H- also known as HoBi-like), and there are subtypes of these genotypes too [41]. In Türkiye, BVDV-1 is found predominantly, BVDV-2 and BVDV-3 types have also been reported [41-46]. The isolates found in our study were found to be the common BVDV-1 genotype and all isolates were clustered in BVDV-1a subtype. According to literature review this is the first time that molecular characterization has been performed in the study location and this report has made an important contribution to the literature in this respect.

There are serological and molecular reports of infection with BRSV and BPIV-3. The seroprevalence of these two viruses and their effect on respiratory problems have been demonstrated [3-5,11,14,33,39,40,47]. Molecularly, BPIV-3 has three genotypes named as genotype A, genotype B and genotype C, while BRSV is divided into eight subgroups. These are I (former subgroup B), III (former subgroup A), II, IV, V and VI (former subgroup AB), subgroup VII, VIII and a ninth subgroup has been proposed [11,14]. Molecular data from previous studies indicate that field isolates are molecularly similar and that different types have not yet been encountered in circulation. In the reported previous studies, genotypes were identified as genotype C for BPIV-3, while BRSV isolates were reported as subgroup III [3,5,11,14, 33]. The results of our study were similar to those of previous studies and BPIV-3 isolates were characterized as genotype C while BRSV isolates were found as subgroup III. Our study made a contribution by characterizing these two viruses for the first time in the study area.

When the vaccines available in our country and possible antibody responses are examined, it is thought that there will be no problem in protecting against BVDV and BRSV strains that are currently circulating in the field in terms of BVD and BRS viruses [32]. However, with BPIV-3, it has been reported that there may be problems in providing adequate protection against genotype C strains since the vaccines in use are genotype A-based [33,47]. The results of the study are similar to this finding and suggest that genotype C-based vaccines should be imported/produced for more effective immunity. Further studies investigating the immune response are needed in this regard. It would be most effective to repeat the studies for all three viruses at regular intervals to determine the new strain situation in the field and to update the control strategies according to the results obtained.

In conclusion, the viral agents BPIV-3 (n=3), BVDV (n=8) and BRSV (n=4) were molecularly detected in cattle with respiratory problems in the Kars region and were found to play a role in the etiology of respiratory diseases. The fact that the majority of cattle with respiratory problems were less than 1 year old indicates that these animals need more attention in terms of protection and control measures. Phylogenetic analysis indicated that BRSV subgroup III, BPIV-3 genotype C and BVDV-1a are the pathogens circulating in the region. It is considered that

these data will help to guide the fight against viral agents that frequently cause respiratory diseases.

Declarations

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

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Ethical Statement: The study has been approved by the ethics committee of Kafkas University (Approval No. KAÜ-HADYEK/2020-031).

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

Declaration of Generative Artificial Intelligence: The article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: EEE, NC: Idea and study design. EEE, MS, AHK, EA: Sample collection; NC, VY: Laboratory analyses; EEE, NC: Manuscript preparation. EEE, NC, AHK, VY, EA, MS have read and approved the final manuscript.

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Research Article

Effect of Osthole on Inflammatory Responses of Mice with Primary Sjögren's Syndrome

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Abstract

We aimed to assess the effect of osthole on the inflammatory responses of mice suffering from primary Sjögren's syndrome (PSS). Fifty naive non-obese diabetic (NOD/Ltj) mice were included to establish a spontaneous model of PSS and assigned into a control group, a 20 mg/kg osthole group, a 30 mg/kg osthole group, a 40 mg/kg osthole group and a 50 mg/kg osthole group in a random manner. Osthole (20, 30, 40, and 50 mg/kg, once a day) together with normal saline (every day) in an equal volume were injected into osthole treatment groups and control group, respectively. Compared with the control group, the inflammatory cells in salivary gland tissues of NOD/Ltj mice manifested gradually ameliorated infiltration, the destruction of acinar cells was relieved gradually ($P<0.05$). In comparison to those obtained from the control group, the inflammatory factors IFN-α, IFN-β, IL-6, IL-8 and TNF-α as well as the GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) in salivary gland tissues of NOD/Ltj mice declined gradually at the expression level as the osthole concentration augmented, showing statistically significant differences ($P<0.05$). Osthole is capable of mitigating inflammatory responses of PSS mice by impeding the cGAS-STING signaling pathway.

Keywords: Osthole, rimary Sjögren's syndrome, inflammatory responses

Introduction

As an autoimmune disease, primary Sjögren's syndrome (PSS) is mainly characterized by symptoms of dryness affecting many organs of the whole body, such as dry mouth, dry eyes, and dry sensation in other parts (such as skin, nose, throat and vagina) [1]. Besides, PSS may also contribute to a series of other symptoms such as joint pain, muscle pain, fatigue, and digestive problems [2]. PSS is mainly ascribed to the decrease in the volume or quality of secretion of glands due to the attack from the immune system, with salivary glands and lacrimal glands as the most common tissues involved [3]. Symptoms of dryness are the major manifestations of PSS, but PSS may also serve as a contributing factor to autoimmune diseases of other systems, such as rheumatoid arthritis and systemic lupus erythematosus [4]. In the clinical treatment of PSS, specific drugs are unavailable currently, and the focus is to relieve symptoms to keep patients comfortable and prevent complications [5]. The regulation of inflammatory response may become one of the important therapeutic strategies for PSS. Inflammation may involve joint tissues, leading to arthritis, and it may also involve muscle tissues, resulting in symptoms such as myalgia and myasthenia [6]. Hence, discovering and developing effective therapeutic drugs in this regard is crucial for PSS patients.

Osthole, a coumarin derivative, exists in several common medicinal plants, such as common Cnidium fruit and Angelica sinensis [7], which can be acquired by extraction and separation from plants or total synthesis. Osthole contains various biological activities such as antineoplastic, cardiovascular protective, neuroprotective, antibacterial, antiparasitic and anti-inflammatory activities [7-10]. Particularly, osthole has been reported to bind multiple

proteins that regulate inflammatory response [11]. Nonetheless, how osthole affects PSS through corresponding molecular regulatory mechanism remains unclear.

Hence, we herein assessed the effect of osthole on inflammatory responses on PSS mice, aiming to provide an experimental foundation for treating PSS.

Materials And Methods

Ethical Approval

This study was conducted after approval by our hospital's animal ethics committee (Approval No. ZHITCWM2022045), and great efforts have been made to minimize the animals' suffering.

Experimental Materials and Apparatus

Male naive non-obese diabetic (NOD/Ltj) mice (6 weeks old, n=50) were bought from the Model Animal Research Center of Nanjing University. Osthole (specification: 1 g/vial, purity: HPLC ≥98%, batch No.: 220508) was acquired from Sichuan Vicky Biotechnology Co., Ltd. (China). The normal saline, bicinchoninic acid (BCA) kits, formaldehyde, skimmed milk powder (5%), paraffin, ethanol, neutral gum, radioimmunoprecipitation assay (RIPA) lysis buffer, Tris-buffered saline-Tween 20 (TBST) and enhanced chemiluminescence (ECL) kits were bought from Solarbio (Beijing, China). A hematoxylin-eosin (HE) staining kit sourced from Beyotime (Shanghai, China). The TRIzol reagent, a PrimeScript reverse transcription (RT) Master Mix kit was offered by TaKaRa (Japan) together with a SYBR® Premix Ex Taq™ quantitative assay kit. A polyvinylidene fluoride (PVDF) membrane was provided by Invitrogen (USA). All antibodies were offered by Abcam. An optical microscope was purchased from Leica (Germany).

Feeding and Grouping of Animals

All male NOD/Ltj mice were adaptively fed for one week in cages (5 mice/cage, humidity: 50-70%, temperature: 22-24°C) with a 12/12 h light/dark cycle. Moreover, enough food and water were offered to ensure that mice could take food and water freely. The Guide for Care and Use of Laboratory Animals was set as the standard for all assays on mice [12]. All treatments started when mice were 8 weeks old: Five groups were determined for equal and random allocation of the 50 NOD/Ltj mice: a control group, a 20 mg/kg osthole group, a 30 mg/kg osthole group, a 40 mg/kg osthole group and a 50 mg/kg osthole group. Mice in the osthole treatment groups were injected with osthole (20, 30, 40, and 50 mg/kg) once a day, while normal saline was injected in an equal volume to those in the control group every day. The salivary volume of mice in each group was measured at Weeks 2, 3 and 4 after treatment. Following 4 weeks of treatment, mice were euthanized to collect the salivary gland tissues for further detection.

Observation of Salivary Gland Tissues by HE Staining

Histological examination was conducted on salivary gland tissue samples of the submandibular gland of mice using the HE staining kit. Briefly, salivary gland tissues were successively subjected to fixation in formaldehyde, dehydration, and embedding in paraffin. Thereafter, paraffin-embedded tissues were sliced into tissue sections (4 μm in thickness) and then stained with HE. Following dehydration with gradient ethanol, the sections were fixed with neutral gum. Finally, the optical microscope was employed to observe the staining results.

Observation of Variations of the Liver

At Week 4 following treatment, the liver was taken out from the euthanized mice, weighed and photographed. Furthermore, HE staining was carried out to observe the morphology of stem cells in liver tissues.

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis of Inflammatory Factor Expressions

Salivary gland tissues collected from each group of mice were ground, followed by qRT-PCR analysis on inflammatory factors to obtain their expression levels by reference to the methods adopted by Zeng et al.[13]. According to the steps in the instructions, total RNA extraction from mouse salivary gland tissues was conducted using TRIzol reagent. Subsequently, the PrimeScript RT Master Mix kit was employed to obtain cDNA from the extracted total RNA through reverse transcription. Afterwards, using following the instructions of the SYBR® Premix Ex Taq™ quantitative assay kit, qRT-PCR was carried out to measure the gene expression levels of IFN-α, IFN-β, IL-6, IL-8 and TNF-α in salivary gland tissues. The internal reference determined as GAPDH, the 2^{-Ct} method was adopted for the calculation of gene relative expressions. *Table 1* presents the sequences of primers utilized.

Measurement of Levels of Cyclic GMP-AMP Synthase (cGAS)-Stimulator of interferon Genes (STING) Signaling Pathway-Associated Proteins by Western Blotting

Salivary gland tissues collected from mice in each group were ground and then detected for the content of cGAS-STING signaling pathway-associated proteins through Western blotting by reference to the methods utilized by Lyu et al.[14]. The isolation of total protein was completed through lysis of the homogenate of salivary glands using the RIPA lysis buffer. Then the content of the isolated total protein was quantified by BCA kit. Next, 10% sodium

Table 1. Sequences of primers used in qRT-PCR

Name	Primers for PCR (5'-3')	
IFN-α	Forward	GCCTCGCCCTTTGCTTTACT
	Reverse	CTGTGGGTCTCAGGGAGATCA
IFN-β	Forward	ATGACCAACAAGTGTCTCCTCC
	Reverse	GGAATCCAAGCAAGTTGTAGCTC
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCAGGTTG
IL-8	Forward	TGTTCACAGGTGACTGCTCC
	Reverse	AGCCCATAGTGGAGTGGGAT
TNF-α	Forward	AGGCACTCCCCCAAAAGATG
	Reverse	CCACTTGGTGGTTTGTGAGTG
GAPDH	Forward	ACAGCAACAGGGTGGTGGAC
	Reverse	TTTGAGGGTGCAGCGAACTT

dodecyl sulfate polyacrylamide gel for protein lysate separation was performed, followed by PVDF membrane transfer. Subsequently, the TBST buffer mixed with 5% skimmed milk powder was added for room-temperature blocking of the PVDF membrane for 1 h. Afterwards, the membrane was added with primary antibodies (anti-cGAS, anti-STING and anti-β-actin) for incubation at 4°C overnight. Following membrane washing, 2 h of incubation with secondary antibodies labeled with horseradish peroxidase was completed. Finally, the ECL kit was used to detect protein signals, with the results analyzed by ImageJ software.

Statistical Analysis

GraphPad Prism 8.0 software was utilized for statistical analysis. For each experiment, three replicates were set. Mean ± standard deviation ($\bar{x}\pm s$) was selected as the format to express all data, and the comparison among groups and pairwise comparison between groups were completed through analysis of variance and LSD-*t* test, respectively. The differences with statistical significance were indicated by $P<0.05$.

Results

Effect of Osthole on Disease Progression of Salivary Gland Tissues in NOD/Ltj Mice

Compared with the control group, both the penetration of inflammatory cells and the destruction of acinar cells in salivary gland tissues of NOD/Ltj mice were gradually ameliorated with the increase in osthole concentration, implying that for NOD/Ltj mice, osthole impedes the disease progression of salivary gland tissues *(Fig. 1)*.

Effect of Osthole on Salivary Volume in NOD/Ltj Mice

In comparison to the control group, the saliva volume secreted by salivary gland tissues rose bit by bit in NOD/Ltj mice with the increase of osthole concentration, with a difference of statistical significance ($P<0.05$) *(Table 2)*.

Effect of Osthole on the Liver of NOD/Ltj Mice

No obvious variations were observed in the texture and weight of the liver of NOD/Ltj mice treated with osthole in comparison to those obtained from the control group ($P>0.05$). Besides, the observation results of liver stem cells of mice in the 50 mg/kg osthole group uncovered that these cells displayed normal morphology. These results demonstrated that osthole has no significant effect on NOD/Ltj mice's liver *(Fig. 2)*.

Role of Osthole in Influencing Inflammatory Factors in NOD/Ltj Mice from the Aspect of Expression Level

The inflammatory factors IFN-α, IFN-β, IL-6, IL-8 and TNF-α gradually dropped at the expression level with the augmentation of osthole concentration, with statistically significant differences by contrast to those in the control group ($P<0.05$). This suggested that osthole represses inflammatory factors in salivary gland tissues of NOD/Ltj mice in terms of their expressions *(Fig. 3)*.

Effect of Osthole on cGAS-STING Signaling Pathway-Associated Protein Levels in NOD/Ltj Mice

When the osthole concentration augmented, the NOD/Ltj mice, contrasted with the control group, exhibited reduced expression levels of cGAS and STING in salivary

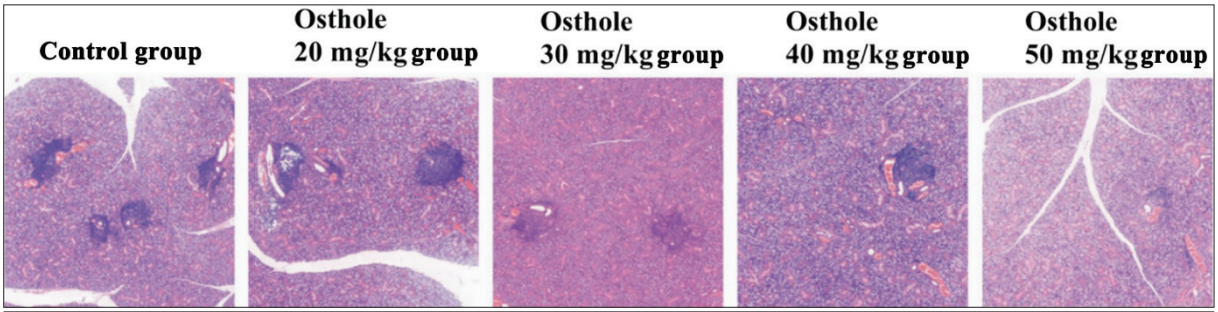

Fig 1. Effect of osthole on disease progression of salivary glands in NOD/Ltj mice. Salivary gland tissues from the submandibular gland in mice observed by HE staining

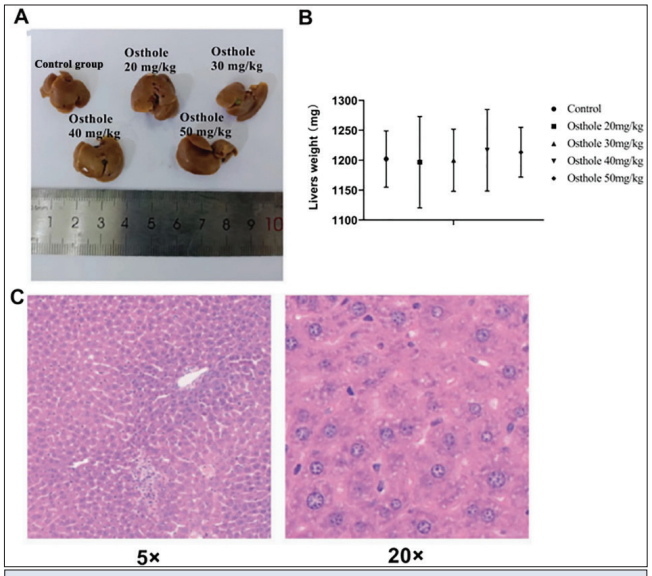

Fig 2. Impact of osthole on NOD/Ltj mice's liver. A-B: Comparison of liver texture and weight of NOD/Ltj mice among groups. C: Histological morphology of liver stem cells in the 50 mg/kg osthole group observed by HE staining

gland tissues in a gradual manner, with differences of statistical significance (P<0.05). This implied that regarding NOD/Ltj mice, osthole inhibits cGAS-STING signaling pathway-associated protein expressions in salivary gland tissues (*Fig. 4*).

Table 2. *Salivary volume of mice ($\bar{x}\pm s$, mg/min)*

Group	n	Week 2	Week 3	Week 4
Control	10	6.21±1.86	6.32±1.52	6.42±1.73
20 mg/kg osthole	10	9.03±1.79^{a}	10.87±1.57^{a}	12.36±1.96^{a}
30 mg/kg osthole	10	11.37±1.64^{ab}	13.56±2.32^{ab}	14.72±1.35^{ab}
40 mg/kg osthole	10	14.13±2.87^{abc}	15.05±2.48^{abc}	17.56±1.78^{abc}
50 mg/kg osthole	10	17.23±2.52^{abcd}	18.86±2.27^{abcd}	19.93±2.07^{abcd}

a *P<0.05 vs. control group,* ***b*** *P<0.05 vs. 20 mg/kg osthole group,* ***c*** *P<0.05 vs. 30 mg/kg osthole group,* ***d*** *P<0.05 vs. 40 mg/kg osthole group*

Discussion

PSS is an autoimmune disease mediated by the immune system, with its pathogenesis involving the abnormal attack on the own tissues from the immune system [15]. In the case of PSS, the attack and destruction from the immune system negatively affect the salivary glands, lacrimal glands and other secretory gland tissues to weaken or eliminate the secretory function of these glands, thus inducing such symptoms of dryness as dry mouth and dry eyes [16]. Inflammatory responses are crucial participants in PSS pathogenesis. After abnormal activation of the immune system, chemokines, cytokines and other inflammatory mediators are delivered, resulting in inflammatory responses. These inflammatory mediators attract and

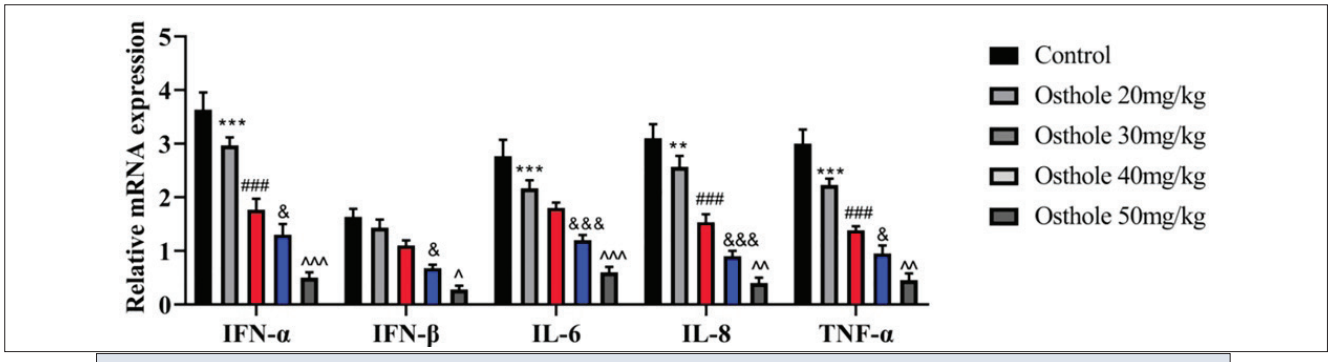

Fig 3. Role of osthole in influencing inflammatory factors in NOD/Ltj mice at the expression level. Expressions of inflammatory factors in murine salivary gland tissues in each group analyzed by qRT-PCR. **P<0.01, ***P<0.001 *vs.* Control Group, $^{###}$P<0.001 *vs.* 20 mg/kg osthole Group, $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 *vs.* 30 mg/kg osthole Group, $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01, $^{\wedge\wedge\wedge}$P<0.001 *vs.* 40 mg/kg osthole Group

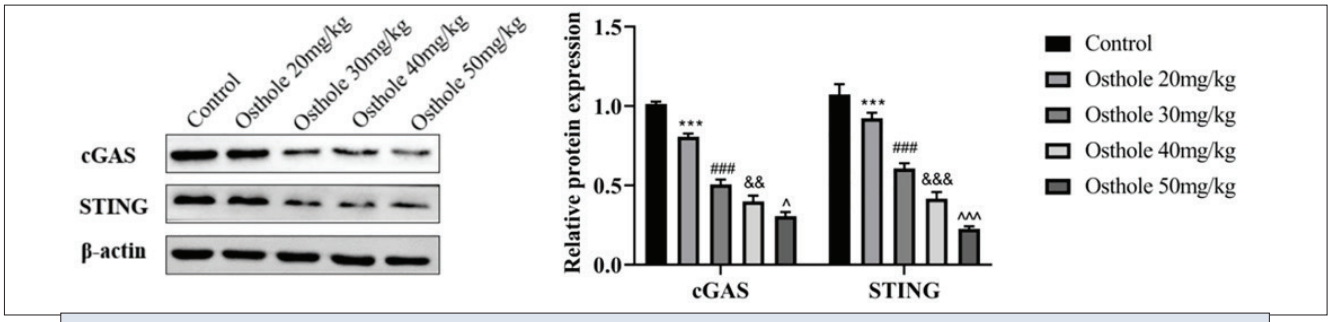

Fig 4. Effect of osthole on cGAS-STING signaling pathway-associated protein content in NOD/Ltj mice. Western blotting for cGAS-STING signaling pathway-associated protein concentrations in salivary gland tissues of mice in each group. ***P<0.001 *vs.* Control Group, $^{###}$P<0.001 *vs.* 20 mg/kg osthole Group, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 *vs.* 30 mg/kg osthole Group, $^{\wedge}$P<0.05, $^{\wedge\wedge\wedge}$P<0.001 *vs.* 40 mg/kg osthole Group

activate inflammatory cells, such as monocytes and lymphocytes, thereby leading to inflammatory responses in local tissues [17]. In the case of PSS, inflammatory responses are mainly observed in the tissues around the affected glands, giving rise to gland damage and hypofunction [18]. In addition to the direct effect on the secretory glands, inflammatory responses may also be responsible for inflammation and injury of other systems and organs, resulting in a series of other symptoms and complications. Accordingly, it is crucial to know the relationship between PSS and inflammatory responses for the purpose of understanding the pathogenesis of PSS, as well as for its diagnosis and treatment.

Liu et al.[19] reported that melatonin repressed the hypofunction and inflammation progression of salivary glands in NOD/Ltj mice by modulating immune response, finally mitigating Sjögren's syndrome-like symptoms. In the present study, 50 NOD/Ltj mice used as spontaneous animal models of PSS were assigned into a control group as well as osthole 20, 30, 40 and 50 mg/kg groups at random. It was uncovered that the destruction of acinar cells relieved in a gradual manner, the infiltration of inflammatory cells in salivary gland tissues of NOD/Ltj mice gradually meliorated, and the volume of saliva secreted by salivary gland tissues rose bit by bit with the increase in osthole concentration, and they were statistically significantly different from those in the control group ($P<0.05$). This suggested the inhibitory effect of osthole on disease progression of salivary gland tissues in NOD/Ltj mice, comparable to the results of Liu et al.[19]. Besides, the osthole treatment groups compared to the control group exhibited no obvious variations in the texture and weight of the liver ($P>0.05$). Furthermore, it was observed that liver stem cells of mice in the 50 mg/kg osthole group displayed normal morphology. These results signified that osthole has no significant effect on the liver of NOD/Ltj mice, with good drug safety.

Osthole has been reported to exert a good anti-inflammatory effect [20]. For instance, a study conducted by Kordulewska et al.[21] manifested that osthole could alleviate gastrointestinal inflammation by impeding pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) from secretion. Moreover, Wang et al.[22] discovered that osthole could modulate the polarization to M1 macrophages induced by lipopolysaccharides, reducing inflammatory responses. In this study, as the osthole concentration augmented, NOD/Ltj mice displayed progressively lowered the expression levels of such inflammatory factors as IFN-α, IFN-β, IL-6, IL-8 and TNF-α in salivary gland tissues compared with the control group, presenting statistically significant differences ($P<0.05$). This verified the ability of osthole to suppress the expression of inflammatory factors in salivary gland tissues of NOD/Ltj mice, echoing research findings by Kordulewska et al.[21] and Wang et al.[22].

As a crucial immune signaling pathway, the cGAS-STING signaling pathway serves as a vital player in resisting pathogenic microorganisms and infections and regulating autoimmune balance [23]. The cGAS-STING signaling pathway under activation can promote immune cells to respond to virus infection and DNA damage, thus regulating inflammatory responses and antiviral immunity [24]. Nevertheless, excessive activation or abnormal regulation of such a pathway may also give rise to such diseases as autoimmune diseases and inflammatory diseases. For this reason, the regulation of balance in this pathway is crucial for the maintenance of immune balance and prevention of diseases [25]. As reported by Zhou et al.[26], the activated cGAS-STING signaling pathway had a close correlation with the pathogenesis of PSS. Rapamycin is found to be able to alleviate submandibular gland lesions in mice with Sjögren's syndrome by repressing the cGAS-STING signaling pathway from activation. It was uncovered through this research that compared to the control group, the elevation of osthole concentration gradually lowered cGAS plus STING expression levels in salivary gland tissues of NOD/Ltj mice, and there were differences with statistical significance ($P<0.05$). This demonstrated that osthole impedes the protein expression associated with the cGAS-STING signaling pathway in salivary gland tissues of NOD/Ltj mice, thereby exerting its anti-inflammatory effect. To sum up, it was discovered in this study for the first time that osthole can mitigate the inflammatory responses of PSS mice by suppressing the cGAS-STING signaling pathway, which renders a novel idea for curing PSS. However, some shortcomings also exist in this research. For example, the effect of osthole was only studied using NOD/Ltj mouse models. Therefore, subsequent clinical studies are required to validate conclusion of this work.

In conclusion, osthole can relieve inflammatory responses in PSS mice by inhibiting the cGAS-STING signaling pathway, suggesting that osthole probably acts as a potential drug effective for PSS.

Declarations

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

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Ethical Approval: This study has been approved by the animal ethics committee of our hospital (approval No. ZHITCWM2022045), and great efforts have been made to minimize the animals' suffering.

Competing Interests: There is no conflict of interest.

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

Authors' Contributions: D. Zhang and J. Yao designed and performed the study, and drafted the paper; J. Li conceived and supervised the study, and significantly revised the paper; L. Jiang, B. Yu, C. Zhou and D. Zhang performed and analyzed the experiments. All authors have approved the submission and publication of this paper.

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Research Article

Adulticidal, Larvicidal, and Repellent Potential of Ethyl Acetate Extract of *Moringa oleifera* against *Rhipicephalus microplus* Cattle Ticks

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Abstract

The emergence of tick resistance against synthetic and eco-toxic chemical acaricides has stressed the need to find target-specific, non-toxic, and more potent alternatives such as botanicals. The current study was designed to investigate the acaricidal and repellent potential of ethyl acetate extract of *Moringa oleifera* seeds against *Rhipicephalus microplus* cattle ticks. Adult immersion test, larval immersion test, and tick climbing repellent assay were carried out to evaluate the acaricidal and repellent potential of *M. oleifera* extract. Five different concentrations (20, 10, 5, 2.5, and 1.25 %) of the ethyl acetate extract of *M. oleifera* were prepared to evaluate the acaricidal and repellent potential. Different parameters like adult tick mortality, larval mortality, egg hatchability, inhibition of egg hatchability, oviposition reduction, reproductive index, reproductive efficiency index, tick repellency, and product effectiveness were determined. Ethyl acetate extract of *M. oleifera* showed 70% adult mortality after 24 h treatment when 20% concentration was used. Similarly, 73% larval tick mortality was observed at 20% concentration. The results also showed the dose-dependent response of ethyl acetate extract of *M. oleifera* seeds against egg hatchability, inhibition of egg hatchability, oviposition reduction, reproductive index, reproductive efficiency index, and tick repellency, confirming that 20% concentration is effective as an acaricide and repellent against *R. microplus*. Therefore, the tested extract can be considered a possible candidate for controlling *R. microplus* in cattle.

Keywords: Acaricides, *Moringa oleifera*, Seed extract, Repellents, *Rhipicephalus microplus*, Ticks, Cattle

Introduction

Rhipicephalus (Boophilus) microplus ticks are hematophagous ectoparasites of cattle *(Bos taurus)* and are mostly found in tropical and subtropical regions [1]. *R. microplus* ticks also infest other bovids including water buffalo *(Bubalus bubalis),* gaur *(B. frontalis),* and banteng *(Bos javanicus)* [2]. *R. microplus* cattle ticks are causing huge economic losses in terms of decreased milk and weight gain, morbidity, mortality, hide damage, and cost of disease control. The economic loss due to *R. microplus* is estimated at 22-30 billion dollars/annum [3]. They also transmit several pathogens including *Babesia bovis*, *Babesia bigemina*, *Anaplasma marginale*, and thrombocytopenia syndrome virus [4]. No doubt, *R microplus* (Acari: Ixodidae) infest livestock animals, but they also have zoonotic potential because they can transmit certain pathogens to humans [5,6]. For example, in the United States of America, 4151 cases of human granulocytic anaplasmosis (HGA) were recorded in 2016, and 2358 cases of babesiosis in 2017 [7,8].

Various chemical drugs including pyrethroids (permethrin, flumethrine), arsenical preparations, chlorinated hydrocarbons (DDT, lindane), organophosphates (coumaphos), carbamates (carbaryl) macrocyclic lactones (ivermectin), formamidines (amitraz), phenyl pyrazoles (fipronil) and insect growth regulators (fluazuron) were used to

control *R. microplus* population [9,10]. Unfortunately, the continuous and irrational use of these chemical acaricides has led to the development of resistance in the tick population [11]. The resistance is developed because of the mediation of para-sodium channels and mutation in the acetylcholinesterase enzyme system [12]. These chemical acaricides are also not readily accessible to the farmers because of their high costs. However, they may have hazardous effects on human and animal health through the contamination of cattle milk and meat [13]. Due to the increasing emphasis on sustainable agriculture and the growing interest in organic farming practices, synthetic acaricides such as pyrethroids, organo-chlorines, and organophosphates have faced restrictions in the global market [14]. Consequently, scientists and researchers have changed their interest in the development of new agents and are focusing on alternative control strategies [15,16]. Therefore, many effective control strategies have been used, including pheromone-assisted control, vaccination, entomopathogenic fungi, nanoparticles, and botanicals [17,18].

Plant-derived products seek special attention among the alternate control strategies and can be promising as an acaricidal source [19]. The insecticidal and acaricidal potentials of these plant extracts are due to the presence of chemical components and their interaction among themselves [20,21]. Furthermore, plant extracts are biodegradable, eco-friendly, and target-specific to the species.

Moringa oleifera is a globally distributed plant and most people use it in their diet because of its therapeutic properties. It has been used to cure various chronic diseases for centuries because of its ethnomedicinal properties [22]. Because of its pharmacological properties, it has been used as an antioxidant, anti-inflammatory, antimicrobial, antiviral, antifungal, anthelmintic, and anti-parasitic [23]. These properties are due to the presence of several chemical compounds such as tannins, flavonoids, saponins, terpenoids, glycosides, steroids, coumarins, proteins, carbohydrates, and starches. Other phytochemical analysis revealed the presence of isoquercetine, astragalin, isorhamnetin, daidzein, apigenin, luteolin, genistein, epicatechin, ferulic acid, gallic acid, ellagic acid, sinapic acid, caffeic acid, chlorogenic acid, salicylic acid, vicenin-2, niazimicin, and niazirin [24].

In this study, we investigated the potential of ethyl acetate extract of *M. oleifera* on adult tick mortality, larval tick mortality, percentage egg hatchability, inhibition of oviposition, reproductive index, and reproductive efficiency index of *R. microplus*. In addition, we also investigated the repellent effect of *M. oleifera* against adult ticks of *R. microplus*.

Materials and Methods

Ethical Approval

This study was conducted with approval from the Departmental Ethical Review Board, Department of Parasitology, University of Agriculture, Faisalabad (Approval No. PAR/315-24, dated 03-06-2024).

Plant Material Preparation

The seeds of *M. oleifera* were purchased from the local herbal market in Karkhana Bazar Faisalabad, Pakistan. The seeds were then identified by an expert botanist at the Department of Botany, University of Agriculture, Faisalabad, Pakistan. The seeds were then air-dried for 48 h and ground into fine powder. The powder was kept in a clean and dry glass container (Imperial-G®). The plant extract was prepared by mixing 300g of *M. oleifera* powder into 2700mL of liquid ethyl acetate (UNI-CHEM®) in a 1:10 w/v ratio. The material was stirred continuously after regular intervals and filtered. The desired extract was obtained by reducing the vacuum under Xinchen®XZ-10L at 27ºC. The extract was then stored at -4ºC until the analysis. The percentage yield was then calculated by the formula given below:

$$\text{Percentage yield} = \frac{weight\ of\ the\ dry\ extract}{weight\ of\ the\ dry\ seeds\ before\ extraction} \times 100$$

Collection and Preparation of Ticks

A total of 300 *R. microplus* ticks were collected from different areas of Tehsil Chuk Chumbra, District Faisalabad, Pakistan. The ticks were collected in 100 mL plastic bottles (Merum®) from different body parts including the neck, groin, and udder of cattle with the help of blunt end forceps (Premium®). The plastic bottles were provided with tiny holes for proper aeration. The ticks were then transported to the Chemotherapy Lab, Department of Parasitology, University of Agriculture, Faisalabad, Pakistan. The ticks were washed and identified under a stereomicroscope (Nikon®) according to the identification guide [25,26]. Two groups of 300 ticks were made. One group contained 210 ticks for the adult immersion test and the remaining 90 ticks were placed in a separate group and incubated in biological oxygen demand (BOD at 27ºC and 85% relative humidity. The latter was used to evaluate, inhibition of oviposition (IO), egg hatchability (EH), reproductive index (RI), and reproductive efficiency index [27]. The effectiveness of the product was also investigated. For these purposes, the following formulas were used.

$$\text{IO (\%)} = \frac{\text{IE control} - \text{IE treated}}{\text{IE control}} \times 100$$

$$\text{EH (\%)} = \frac{\text{Number of eggs hatched}}{\text{total number of eggs}} \times 100$$

$$RI\ (\%) = \frac{\text{Weight of eggs laid}}{\text{Weight of adult females before treatment}} \times 100$$

$$REI = \frac{\text{egg mass weight} \times \%\ \text{egg hatching}}{\text{engorged female tick weight}} \times 20000$$

$$PE\ (\%) = \frac{\text{RE control} - \text{RE treatment}}{\text{RE control}} \times 100$$

Bioassays

Adult immersion test, larval immersion test, and tick repellent assay were used to evaluate the acaricidal and repellent potential of ethyl acetate extract of *M. oleifera* seeds.

Adult Immersion Test (AIT)

The adult immersion test was carried out to determine the adult engorged *R. microplus* tick mortality. A total of 210 engorged female cattle ticks were divided into seven subgroups which include five subgroups for five different concentrations along with positive (cypermethrin 0.1% of K-CYPER®) and negative (distilled water) control groups. Each subgroup was further subdivided into three replications and each replication contained 10 ticks. Five concentrations (20, 10, 5, 2.5, and 1.25) of *M. oleifera* were prepared. 5 mL of each concentration was taken out in separate Petri dishes (SMART LAB®). The collected ticks were then immersed, removed, washed with distilled water, and placed on filter paper to dry. All dried ticks were then transferred into a desiccator and placed in a BOD incubator where temperature and relative humidity were maintained at 27ºC and 85% respectively. The adult tick mortality of all groups was calculated after 6, 12, and 24 h treatment. The mortality of *R. microplus* ticks was confirmed by the loss of motility or sensitivity and the absence of motion of malpighian tubules and pedal reflex.

Percentage mortality was calculated by the following formula:

$$\text{Percentage mortality} = \frac{Number\ of\ dead\ ticks}{Total\ number\ of\ ticks} \times 100$$ [28]

Larval Immersion Test

The LIT was used to evaluate the larval mortality [29]. Similar to the adult immersion test five concentrations (20, 10, 5, 2.5, 1.25%) concentrations were taken in 1.5 mL microcentrifuge tubes. 100 *R. microplus* larvae were placed in each 1.5 mL microcentrifuge tube. The positive control group was treated with 1 mL of 0.1% cypermethrin while the negative control was treated with distilled water. After adding the larvae, the tubes were sealed and shaken vigorously for 30 sec and then gently for 5 min. Each treated larva was then transferred to filter paper to dry. After that, the larvae were placed on Whatman filter paper No. 1 (8.5×7.5 cm), folded, and sealed with hairpins to form a packet. These packets were then incubated at 27°C and 85% relative humidity. Live and dead larvae were counted after 48 h treatment. The experiment was repeated three times and larval mortality was counted by the following formula:

$$\text{Percentage mortality} = \frac{Number\ of\ dead\ larvae}{Total\ number\ of\ larvae} \times 100$$ [30]

Tick Climbing Repellent Assay (TCRA)

The TCRA was used to investigate the repellent activity of ethyl acetate extract of *M. oleifera*. The same protocol was followed as adapted by [31]. For this procedure, two vertical aluminium rods were taken which were spaced 7cm from each other and attached to an aluminium base. Each aluminium rod was encased in a glass tube with a piece of filter paper wrapped around it. The top of each tube was sealed with wet cotton wool. *M. oleifera* concentrations (20, 10, 5, 2.5, 1.25 %) were applied to aluminum rods and 30 ticks were placed for each concentration. A similar procedure was done for positive (0.1% cypermethrin) and negative control (distilled water). Some ticks were unable to climb up due to the repellent effect of ethyl acetate of *M. oleifera* and data were recorded for all groups. The percentage repellency was calculated by using the following formula:

$$\text{Percentage Repellency} = \frac{100 - (\text{mean number of ticks not repelled})}{\text{mean number of ticks not repelled by control}} \times 100$$

Statistical Analysis

Results of *R. microplus* adult cattle ticks (adult tick mortality, larval mortality, reproductive index, reproductive efficiency, inhibition of oviposition, percentage larval hatching, and product effectiveness) were analyzed using Tuckey's test ($P>0.05$). Lethal concentrations (LC_{50} and LC_{90}) and repellent concentrations (RC_{50} and RC_{90}) were calculated by using Statistical Product and Service Solutions (SPSS) software.

Results

Yield of the Ethyl Acetate Extract of *M. oleifera*

Using the hydrodistillation process, a yield of 12.8% was determined for the ethyl acetate extract of *M. oleifera*.

Adult Tick Mortality

Effect of Ethyl Acetate Extract of M. Oleifera Seeds on Adult Tick Mortality: The results of the adult immersion test for various concentrations (20, 10, 5, 2.5, 1.25%) of ethyl acetate of *M. oleifera* seeds are shown in *Table 1* and *Fig. 1*. Value for adult tick mortality was lowest at AX=1% concentration whereas 70% mortality was seen at EX=20% treatment. The results demonstrated that a 20% concentration of *M. oleifera* showed a significant ($P<0.05$) acaricidal effect against *R. microplus* adult ticks when compared with negative control. LC_{50} and LC_{90} values were calculated as shown in *Table 2*.

Table 1. *Percentage mortality of adult Rhipicephalus microplus ticks treated with various concentrations of ethyl acetate extract of M. oleifera seeds under laboratory conditions (27°C and RH = 85%)*

Concentrations of *M. oleifera*	Mortality After 6 h	Mortality After 12 h	Mortality After 24 h
AX	10 ± 0^{d}	16.6 ± 5.77^{c}	23.33 ± 5.77^{d}
BX	23.33 ± 5.77^{cd}	33.33 ± 5.77^{c}	43.33 ± 5.77^{d}
CX	36.66 ± 5.77^{cd}	46.66 ± 5.77^{c}	56.66 ± 5.77^{cd}
DX	46.66 ± 5.77^{bc}	56.66 ± 5.77^{bc}	66.66 ± 5.77^{bc}
EX	53.33 ± 5.77^{b}	63.33 ± 5.77^{b}	70 ± 10^{b}
PC	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
NC	0 ± 0^{e}	0 ± 0^{d}	0 ± 0^{e}

AX: *M. oleifera 1.25%;* ***BX:*** *M. oleifera 2.5%;* ***CX:*** *M. oleifera 5%;* ***DX:*** *M. oleifera 10%;* ***EX:*** *M. oleifera 20%;* ***PC:*** *Positive Control;* ***NC:*** *Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other*

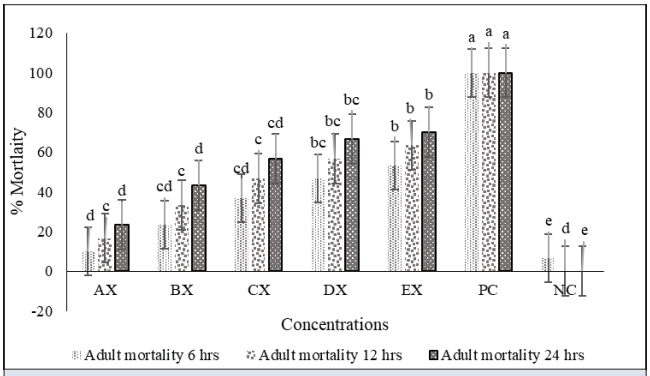

Fig 1. Effect of different concentrations of *M. oleifera* on adult *R. microplus* ticks. AX: *M. oleifera* 1.25%; BX: *M. oleifera* 2.5%; CX: *M. oleifera* 5%; DX: *M. oleifera* 10%; EX: *M. oleifera* 20%; PC: Positive Control; NC: Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other

Table 2. *LC_{50} and LC_{90} values with 95% Confidence interval (C.I) of ethyl acetate extract of M. oleifera seeds against adult tick mortality at different time intervals*

	Time Interval	LC50 (95% C.I)	LC90 (95% C.I)	Slope ± SE	X^2	R^2
***M. oleifera* Seed Extract**	6 h	0.057 (0.019-1.070)	6.246 (3.019-8.302)	0.91±0.62	0.176	0.947
	12 h	0.047 (0.017-1.009)	3.721 (1.171-5.223)	1.12±0.69	0.471	0.909
	24 h	0.019 (0.007-0.461)	0.702 (0.039-3.121)	1.68±0.91	0.588	0.886

LC: *lethal concentrations;* ***X^2:*** *Chi-square;* ***R^2:*** *coefficient of determination;* ***SE:*** *standard Error*

Larval Mortality

Effect of Ethyl Acetate Extract of M. oleifera Seeds on Larval Mortality: The larvicidal effect of ethyl acetate extract of *M. oleifera* seeds at various concentrations (20, 10, 5, 2.5, 1.25%) was evaluated against the larvae of *R. microplus* ticks. The results demonstrated that a 20% concentration of *M. oleifera* showed a significant (P<0.05) effect when compared with negative control. LC_{50} and LC_{90} values were also calculated which showed toxicity at 12.41 and 2.41% doses respectively. *Fig. 2* indicates that as extract concentration increased, larval mortality also increased.

Effectiveness of the *M. oleifera* Extract

The efficacy of ethyl acetate extract of *M. oleifera* seeds against *R. microplus* ticks was determined through the product effectiveness parameter. Different concentrations of ethyl acetate of *M. oleifera* seeds were used and proved to have different results concerning effectiveness against

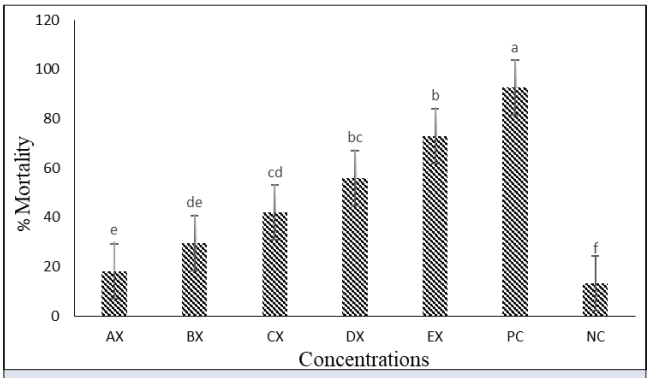

Fig 2. Effect of different concentrations of *M. oleifera* on larvae of *R. microplus* ticks. AX: *M. oleifera* 1.25%; BX: *M. oleifera* 2.5%; CX: *M. oleifera* 5%; DX: *M. oleifera* 10%; EX: *M. oleifera* 20%; PC: Positive Control; NC: Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other

Table 3. *Mean values of egg hatchability, % inhibition of hatchability, oviposition reduction, reproductive index, and reproductive efficiency index engorged females of R. microplus treated with various concentrations of ethyl acetate extract of M. oleifera seeds under laboratory conditions (27°C and RH = 85%)*

Concentrations of *M. oleifera*	Egg Hatchability	%Inhibition of Hatchability	Oviposition Reduction	Reproductive Index	Reproductive Efficiency Index
AX	80.55±1.50^{a}	19.44±1.50^{a}	54.73±0.90^{d}	33.59±0.71^{b}	54.12±2.05^{b}
BX	75.11±3.20^{a}	24.88±3.20^{a}	62.87±1.70^{cd}	27.54±1.26^{bc}	41.4 ±3.64^{bc}
CX	63.11±5.27^{b}	36.88±5.27^{b}	69.05±1.64^{cd}	22.96±1.21^{bc}	29.07±3.92^{cd}
DX	42.44±1.67^{c}	57.55±1.67^{c}	79.94±4.92^{bc}	14.88±3.65^{cd}	12.71±3.55^{de}
EX	27.22±2.54^{c}	72.77±2.54^{c}	89.15±1.74^{b}	8.04±1.29^{d}	4.4±1.04^{ef}
PC	13.11±2.77^{d}	86.88±2.01^{77d}	93.06±10^{a}	5.14±0.74^{e}	1.34±0.32^{f}
NC	88.22±2.52^{a}	11.77±2.52^{a}	40.66±0.95^{e}	44.03±0.7^{a}	77.7±3.36^{a}

AX: *M. oleifera 1.25%;* ***BX:*** *M. oleifera 2.5%;* ***CX:*** *M. oleifera 5%;* ***DX:*** *M. oleifera 10%;* ***EX:*** *M. oleifera 20%;* ***PC:*** *Positive Control;* ***NC:*** *Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other*

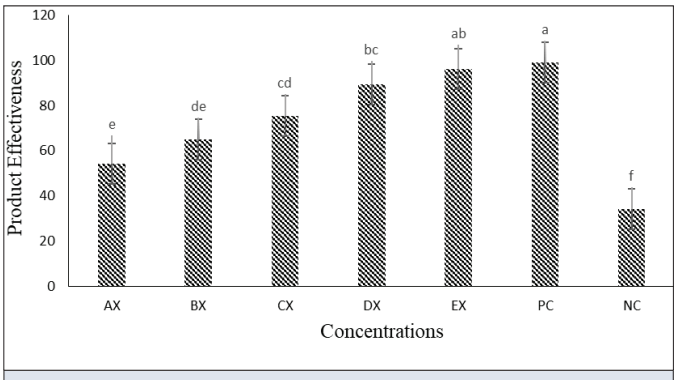

Fig 3. Effectiveness of different concentrations of *M. oleifera* against *R. microplus* ticks. AX: *M. oleifera* 1.25%; BX: *M. oleifera* 2.5%; CX: *M. oleifera* 5%; DX: *M. oleifera* 10%; EX: *M. oleifera* 20%; PC: Positive Control; NC: Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other

R. microplus cattle ticks. The 20% concentration showed significant (P<0.05) results when compared with the negative control and had non-significant (P>0.05) results from the positive control (0.1% cypermethrin) as shown in *Table 3* and *Fig. 3*.

Percentage Repellency

The results of tick repellency of ethyl acetate extract of *M. oleifera* seeds at different concentrations (20, 10, 5, 2.5, 1.25%) were shown in *Table 4* and *Fig. 4*. Tick repellency was calculated at 4 h and 8 h post-treatment. The results

Table 4. *Percentage Repellency of adult Rhipicephalus microplus ticks treated with various concentrations of ethyl acetate extract of M. oleifera seeds under laboratory conditions (27°C and RH = 85 %)*

Concentrations of *M. oleifera*	Repellency After 4 h	Repellency After 8 h
AX	19.62±1.69^{de}	14.94±2.13^{d}
BX	34.81±5.59^{d}	29.89±6.4^{c}
CX	50.74±4.62^{c}	43.05±2.22^{c}
DX	64.81±3.39^{bc}	49.1±4.89^{b}
EX	77.4±4.2^{b}	60.49±4.04^{b}
PC	97.77±2.22^{a}	91.45±4.31^{a}
NC	7.4±2.79^{e}	4.27±2.82^{e}

AX: *M. oleifera 1.25%;* ***BX:*** *M. oleifera 2.5%;* ***CX:*** *M. oleifera 5%;* ***DX:*** *M. oleifera 10%;* ***EX:*** *M. oleifera 20%;* ***PC:*** *Positive Control;* ***NC:*** *Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other*

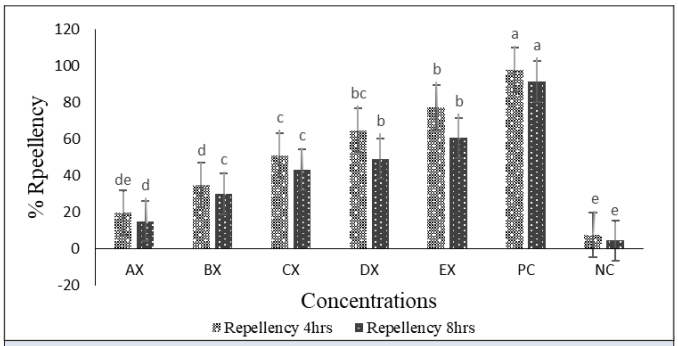

Fig 4. Effect of different concentrations of *M. oleifera* on repellency of *R. microplus* ticks after 4 and 8 h interval. AX: *M. oleifera* 1.25%; BX: *M. oleifera* 2.5%; CX: *M. oleifera* 5%; DX: *M. oleifera* 10%; EX: *M. oleifera* 20%; PC: Positive Control; NC: Negative Control. Mean±SD along with the same superscripts have a non-significant difference (P>0.05) from each other

revealed that the highest repellencies 77.4 and 60.49% were observed after the 4th and 8th h of treatment respectively when a 20% concentration of the extract was used.

Discussion

Livestock have significant economic importance worldwide. Various synthetic chemical drugs have been used to control *R. microplus* but the continuous, frequent, misuse of these drugs has led to the development of *R. microplus* resistance [11]. Scientists are moving towards alternate sources such as vaccination, entomopathogenic fungi, nanoparticles, and botanicals [32]. Due to their pharmacological properties, botanicals have been extensively used as antioxidants, antibacterials, antivirals, antifungals, and antiparasitic [33-35]. Research on plant extracts revealed their insecticidal and acaricidal potential and they could be used to control ticks as an alternative replacement for synthetic compounds [36-38].

M. oleifera plant is widely distributed and mostly present in South Asian countries like Pakistan, India, and Bangladesh. In different countries, it is often used for medicinal purposes, mainly as a laxative and analgesic. These properties are due to the presence of tannins, phenolics, flavonoids, starch, carbohydrates, fats, vitamins, alkaloids, and minerals [39]. The present study was conducted to evaluate the *in vitro* acaricidal and repellent potential of ethyl acetate extract of *M. oleifera* seeds on *R. microplus* engorged females and larvae. Adult and larval immersion tests were carried out against *R. microplus* ticks and results showed maximum adult (70%) and larval mortality (73%) at 20% concentration. The LC_{90} values were also calculated which showed that 6.246, 3.721, and 0.702% values are toxic at 6, 12, and 24 h for adult tick mortality. The present study was in line with the same study investigated by Radwan et al.[40] that demonstrated the acaricidal potential of *Aloe vera* and *Rheum rhabarbarum* against *R. microplus*. A similar study was conducted by Alborzi et al.[41], in which the acaricidal potential of *M. oleifera* against *Hyalomma dromedarii* ticks was evaluated. The results also showed that the hydroethanolic extract of *M. oleifera* caused the mortality of adults and larvae and reduced the ability of ticks to lay eggs.

Our study also revealed the detrimental effects of ethyl acetate extract of *M. oleifera* seeds against reproductive parameters. It is evident by decreased egg-laying ability, egg weight, and reproductive index. Egg hatchability and

reproductive efficiency index have also been reduced. This decrease in reproductive parameters is associated with the presence of phytochemicals in *M. oleifera*. These phytochemicals act synergistically on the salivary and reproductive glands of ticks and act as antitick agents [42]. Different studies confirmed the presence of a variety of phenolics and flavonoids in *M. oleifera* which include syringic acid, caffeic acid, p-coumaric acid, benzoic acid, vallic acid, gallic acid, luteolin, galanin, quercetin, and kamferol. Bustos-Baena et al.[43] confirmed that caffeic acid and vallic acid obtained by *Randia aculeata* had been found effective against *R. microplus* and they are involved in inhibiting the synthesis and functioning of different hormones critical for the reproduction process, leading to decreased egg production and decreased egg hatchability. Zhang et al.[44] investigated the potential of gallic acid against *Ectropis obliqua* and found its application as an anti-insect agent. In a similar study, Baz et al.[45] determined the fumigant and adulticidal effect of garlic, mustard, radish, and rosemary against insects and found them to be an anti-insect agent. The previous study also demonstrated that quercetin present in plant extracts can target specific proteins in insects and hinder their feeding activity [46]. The phenolics particularly quercetin in plant extracts can bind with different amino acids at specific sites and denature the protein structure, hence leading to the disintegration of cuticular membrane [47]. The previous studies also revealed that the phenolics such as vallic acid and ferulic acid caused oxidative stress by the production of superoxide free radicals which have deleterious effects on the growth and development of the insect [48]. In another study, it was confirmed that the application of the phenolics to the insects particularly in ticks produced oxidative stress markers (lipid peroxide, hydrogen peroxide, and superoxides) that are involved in producing oxidative stress and hence act as anti-tick agents. Guneidy et al.[49] confirmed the effects of phenolics, flavonoids, and tannins on tick cuticles and their penetration into the cell membranes to create vacuum and dissociation.

RC_{50} and RC_{90} values were also calculated which showed a repellent effect after 4 and 8 h treatment. The repellent activities of *M. oleifera* may be due to the chemicals that produce a vapor barrier around the insects and prevent the insects from touching or settling on the skin. The repellent effect of plant extracts decreases with time, as results show that it is higher 4 h after treatment and lower 8h after treatment. Based on the evidence provided it is justified that phytochemicals are responsible for anti-tick activities.

The results of the current study confirmed that the ethyl acetate extract of *M. oleifera* seeds has acaricidal and repelling effects against the *R. microplus* ticks. However, animal trials are advised before endorsing the use of this *M. oleifera* extract in practical application. Further study and better techniques are also required to determine the exact chemical composition of *M. oleifera*. In addition, better and improved techniques may increase the yield and residual life of plant extracts.

Declarations

Availability of Data and Material: Data may be available on demand if the reader requires it.

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Ethical Approval: This study was conducted with approval from the Departmental Ethical Review Board, Department of Parasitology, University of Agriculture, Faisalabad (Approval No. PAR/315-24, dated 03-06-2024).

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Research Article

In Vitro and *In Vivo* Anticancer, Anti-inflammatory, and Antioxidant Activity of Dhimran *(Ocimum forsskaolii* benth) Extract and Essential Oil on Carbon Tetrachloride-Induced Hepatotoxicity in Mice

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Abstract

Medicinal plants are rich in bioactive components, which exert various biological activities that are beneficial for living. This study evaluated the antioxidant, antitumor, and anti-inflammatory activities of Dhimran *(Ocimum forsskaolii* benth) in mice. The main volatile compounds in Dhimran essential oil (DEO) were endo-fenchol, tau-cadinol, and β-atlantol, while Dhimran extract (DE) was rich in caffeic acid and quercetin. These active compounds in DE and DEO possess antioxidant activity, scavenging 93% of DPPH radicals, antibacterial activity against MDR bacteria, and anticancer activity against HepG2 cancer cell line. These activities influenced the mice's health. A total of 180 mice were divided into six treatment groups for 30 days as follows**:** T1 was fed a basal diet; T2 received CCl_4 (187 mg/kg BW); T3 received DEO (4 mL/kg); T4 received DE (4 mL/kg); T5 received DEO+CCl_4; and T6 received DE+CCl_4. The oral administration of CCl_4 to mice resulted in an increase in absolute liver weight (ALW) and relative organ weight (ROW) and a significant decrease in body weight gain and feed conversion ratio (FCR). Additionally, there was a notable reduction in levels of red blood cells (RBCs), hematocrit (Ht), hemoglobin (Hb), and platelets; however, there was an increase in white blood cells (WBCs). The administration of CCL_4 in mice lowered total protein content; however, it raised the activity of AST, ALT, ALP, and LDH in mice liver homogenate compared to the control ($P<0.05$). CCl_4 increased inflammation cytokines (TNF-α and IL-6). The coadministration of DEO or DE with CCl_4 significantly corrected all biomarkers to control levels, with a preference for DE. Liver sections from the control, DE, and DEO groups revealed a normal structure. It is concluded that Dhimran extract or essential oil has antioxidant and anti-inflammatory properties that can be used as hepatoprotective agents against CCl_4 toxicity.

Keywords: Anticancer, Antioxidant, Anti-inflammatory, CCl_4 Toxicity, Essential oil, Hepatoprotective, *Ocimum forsskaolii* benth

Introduction

The nutritional, pharmacological, and antioxidant properties of numerous *Ocimum* species plants provide them with substantial commercial and therapeutic benefits [1]. The sweet basil "*Ocimum basilicum*" is the most nutritious. It is widely dispersed and employed in the perfumery and dietary supplement industries [2]. Sharmin et al.[3] conducted numerous studies on extracts of essential oils (EOs) and *Ocimum* species, demonstrating powerful antitumor and antimicrobial properties. The apoptosis of HeLa cells was significantly increased by caffeic acid, isolated from *Ocimum gratissimum* L. It had apoptotic properties on human cervical cancer cells (HeLa) through activating numerous caspases [4]. Ursolic acid also affected microtubules and F-actin [5]. *Ocimum* species are rich in volatile organic components, i.e., phenylpropanoids, monoterpenes, and sesquiterpenes, which exhibit substantial antimicrobial activity against microbes [6].

Ocimum forsskaolii benth, which is named locally in Saudi Arabia as Dhimran is an aromatic medicinal plant belonging to the family *Lamiaceae* that grows in Saudi Arabia and is represented by 26 genera and about 70 species in Saudi Arabia [7]. Native Saudi Arabians have long used it to enhance the flavor of butter and tea and its therapeutic benefits [8]. Traditionally, it is employed as an insect repellent in Eretria, as a fever-reducing agent in Yemen, and for treating eye infections in Rwanda [8]. This plant has exhibited several biological

actions, including local anesthetic [9], antiepileptic, anti-ulcer, and anti-inflammatory properties [10]. Furthermore, it has antibacterial, antioxidant, and cytotoxic properties [8].

The chemical composition of *Ocimum forsskaolii* essential oil varies depending on its geographical origin. The main constituents are estragole (59.4-65.2%) and linalool (25.0-28.1%) [11]. The Ethiopian sample was found to have high levels of (E)-methyl cinnamate or myrcene and methyl chavicol [12]. Sharmin et al.[3] found that the aqueous extract of *Ocimum forsskaolii* leaves contained silver nanoparticles that exhibited antibacterial properties. The primary categories of chemicals in the *Ocimum* include phenylpropanoids, monoterpenes, and sesquiterpenes [13].

A recent *in vitro* study revealed that *Ocimum forskolin* essential oils exhibited *in vitro* anticancer and antibacterial properties. This plant inhibits the growth of cancer cells by conducting tests to measure cytotoxicity. Furthermore, the study validated the oil's impact on specific molecular targets using advanced techniques. Furthermore, the antimicrobial activity of the *Ocimum forsskaolii* essential oil was evaluated against both Gram-positive and Gram-negative bacteria, i.e., *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* [14].

Additionally, the previous studies stated that essential oil derived from *O. basilicum* seeds exhibited significant anticancer effects against Hep3B and MCF-7 with IC_{50} ranging between 56.23 and 80.35 µg/mL) cells, surpassing the efficacy of the positive control, Doxorubicin. Furthermore, the essential oil had strong antibacterial effects against *Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Proteus mirabilis,* and *Pseudomonas aeruginosa*. It also shows potent antifungal activity against *Candida albicans*. The oil had an intense antioxidant action [15].

Carbon tetrachloride (CCl_4) is an artificial chemical compound that has found primary application in the synthesis of chlorofluorocarbons, albeit also being utilized as a pesticide and cleansing agent. As a result of its toxic effects, its production is currently restricted. Carbon tetrachloride's high volatility and environmental persistence lead to its build-up in the atmosphere and groundwater. The general population might be exposed to harmful substances mainly by drinking polluted water and inhaling contaminated air. Elevated substance levels can harm the respiratory, hepatic, central nervous, and renal systems. Tetrachloride carbon is considered potentially carcinogenic to humans [16].

Furthermore, research has shown that basil possesses a diverse range of pharmacological properties that can be applied to several conditions, such as brain injury [17], liver fibrosis, Type 2 diabetes allergies, and anemia [18]. Furthermore, the simultaneous administration of deltamethrin and *Ocimum basilicum* protects against oxidative stress caused by Hg and Cd in an in vivo model of acetaminophen-induced liver injury in Wistar rats [19]. Teofilović et al.[20] demonstrated the effect of basil extract on the markers of oxidative stress, biochemical biomarkers, and morphology of liver injury. The extract improved the efficacy of antioxidant enzymes and decreased the oxidation of macromolecules. The animals treated with basil extract had significantly reduced levels of AST, ALP, and ALT in their blood compared to the control group. There are no studies on the *in vivo* effect of Dhimran extract or essential oils on the CCl_4 toxicity on rats; Hence, this study evaluated the in vitro and in vivo antioxidant, anti-inflammatory, and anticancer activities of Dhimran *(Ocimum forsskaolii* benth) essential oils and extracts in CCl_4-induced hepatotoxicity in mice.

Materials and Methods

Ethical Approval

The Ethical Committee of King Abdulaziz University, Faculty of Medicine, accepted the study with ethical approval reference No. 516-23.

Plant Materials and Preparation of Dhimran Extract (DE)

Ocimum forsskaolii benth plant was collected at Al-Shafa area in Taif City, Saudi Arabia, in June 2023. The plant was identified and authenticated by Dr. Faraj Al-Ghamdi (taxonomist at King Abdulaziz University Herbarium). A plant was deposited at King Abdulaziz University Herbarium, Faculty of Sciences, King Abdulaziz University, Kingdom of Saudi Arabia; the reference specimen is (A. Harbi, 708). The Dhimran essential oil (DEO) was purchased from Aldousiah Farm, Al Bahah, Saudia Arabia.

The Dhimran leaves were dried in the shade and ground into a fine powder. A 10 g of the flour was dispersed in ethanol (70%) and stirred overnight (200 rpm, 25°C). The ethanol was separated by rotary evaporation, and the residues were freeze-dried, and 1 gm was obtained to prepare the different concentrations [21].

GC-MS Analysis

The GC-MS analysis was performed using a Trace GC Ultra/ISQ Single Quadrupole MS and TGSMS Fused Silica Capillary Column (30 m, 0.25 mm, 0.1 mm Film thickness) manufactured by Thermo Scientific, USA. An electron ionization apparatus with a constant flow rate of 1 mL/min and an ionization energy of 70 eV served as the carrier gas for GC-MS detection. The MS injector and transfer line were maintained at a constant temperature of 280°C.

The oven was set to increase its temperature from an initial value of 40°C and gradually raised to 280°C. A percentage relative peak area was utilized to investigate the identified components. The volatile compounds were tentatively identified by comparing their respective retention periods and mass spectra with those of different Library data of the GC/MS system [22].

Phenolic Compounds Content

The total phenolic compounds in DEO and DE were estimated using Folin-Ciocalteu method as Abd Elkader et al.[23]. The phenolic compounds and flavonoids were detected using an HPLC Shimadzu series (Shimadzu, Japan) and a UV-Vis DAD for HPLC analysis. Polyphenols were separated using a separation Gemini column (C18, 150x4.6 mm 3 µm), (Phenomenex, USA) at 0.5 mL/min flow rate and 5°C temperature. The DE was dissolved in a methanol-water mixture (50:50) and injected into the autosampler. All chemicals used in this investigation were of HPLC grade; phosphoric acid (mobile phase B) and acetonitrile (mobile phase A) were combined with Water (Carlo Erba, Germany) to obtain a pH of 2. In addition to a sixty-min total runtime, the gradient elution percentage was altered as follows: a) Initial 5% A and 95% B; b) 35% A and 65% B for 15 min; c) 35% A and 65% B for 20 min; d) 40% A and 60% B for 30 min; e) 40% A and 60% B for 35 min; f) 50% A and 50% B for 40 min; g) 70% A and 30% B for 52 min; and h) 60 min. Examining the UV-Vis spectra of various phenolic standards, 210, 280, and 360 nm were chosen for subsequent HPLC-DAD analysis in the present investigation [24].

The Biological Activities

The Scavenging Activity: The scavenging power of DEO and DE was measured according to Jia et al.[25]. The essential oils and extract at concentrations of (50, 100, and 200 µg/mL) were mixed with DPPH (Sigma, USA) solution and incubated for 30 min at room temperature in the dark. The resultant mixture's absorbance was subsequently quantified at 517 nm. % AA was calculated using the following equation:

$$\% \text{ Scavenging activity} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \text{ x } 100 \quad (1)$$

Antibacterial Activity: The antimicrobial activity of DEO and DE was assessed using the disc diffusion assay, following the methodology of Singh et al.[26], with specific modifications for basil EOs. The McFarland standard solution (0.05) was made by mixing (0.5 mL) $BaCl_2$ and (99.5 mL) H_2SO_4 to obtain a solution with equivalent turbidity to 1.5×10^8 (CFU mL^{-1}) cell density. 200 µL of each bacterial and fungal culture *(L. monocytogenes*, *S. aureus*, *C. jejuni*, and *S. typhi*) was spread on nutrient agar plates. Six mm discs impregnated with varying DEO and DE concentrations were added to plates. The antibacterial activity was quantified by measuring the width of the inhibitory area during a 24-h incubation at 37°C.

Cytotoxic Impact on HepG2 Cells: Cell viability was assessed by measuring their ability to convert the yellow dye MTT to a purple formazan, a process dependent on healthy mitochondria. HepG2 cells were suspended in RPMI 1640 media with 1% antibiotic antimycotic combination (10.000 U/mL potassium penicillin, 10.000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B), as well as 1% L-glutamine at 37°C under 5% CO_2 using a water-jacketed carbondioxide incubator (Sheldon, TC2323, Cornelius, OR, USA).

Around 10.000 liver cancer cells were seeded per well in fresh media (2 mL) on 96-well plates. The microtiter plate was incubated for a day at 37°C and CO_2. The medium was replaced with a fresh one supplemented with FBS and varying concentrations of DE or DEO (50, 100, and 200 µg/mL), then incubated for two days [27]. The medium was aspirated after 48 h of incubation. Then, 40 µL of MTT salt (2.5 µg/mL) was added to each well, and the cells were further incubated for 4 hat 37°C with 5% CO_2. To end the reaction and dissolve crystals, 200 µL of 10% SDS in deionized water was added to each well and incubated overnight at 37°C. A known cytotoxic natural substance (100 µg/mL) was a positive control, causing complete cell death under the same conditions. The cells were collected using a trypsin-EDTA buffer and treated with trypan blue to distinguish viable cells. The live cell count was determined, and the findings were presented as the percentage of inhibition of liver cancer cell lines [28]. A microplate reader (Bio-Rad, USA) was used to measure the absorbance at 595 nm to evaluate the effects of DEO in DMSO on cell viability.

$$\%viability = \frac{Reading\ of extract}{Reading\ of\ negative\ control} \times 100 \quad (2)$$

Animal and Experimental Design

A total of 180 mice weighted (30-42 g) were obtained from the breeding animal house, adapted, and kept under full hygienic conditions. The plastic boxes were subjected to a 12-h dark-light cycle, 40-60% relative humidity, and a temperature of 23.2°C. They delivered water and rats' diet at their discretion throughout the experiment [29]. The mice were given two weeks to acclimate to the experimental animal laboratory setting. The accommodation and administration of the animals and the experimental protocols were conducted per the principles delineated in the Guide for the Care and Use of Lab Animals following the National Committee of Bioethics (NCBE 2023). After the accommodation period, the animals were weighed. Randomly, ten mice were allocated to six groups: The first group was fed a basal diet, the second group was delivered

carbon tetrachloride (CCl_4, 187 mg/kg BW, 1/10 LD50), the third group delivered Dhimran essential oil (DEO, 4 mL/kg), the fourth group received Dhimran extract (DE, 4 mL/kg), the fifth group received DEO+CCl_4, and the sixth group delivered DE+CCl_4. The mice received their daily oral dosing for one month.

Determination of Weight Gain and Organ Weight

The growth performance parameters of mice groups were calculated following Zhou et al.[30]. At the end of the experiment, the animals were decapitated from the cervical region. Following the dissection of the heart, brain, liver, kidney, lung, and spleen, excess fat was eliminated, and the percentages of the relative weight of organs were calculated.

Blood Biochemistry

Sample Collection and Preparation: At the end of 28 days, the mice were fasted overnight, then were slaughtered via jugular vein severance, and two blood samples were obtained. The first 0.5 mL sample was gathered in an EDTA tube for hematological examination. The other part (2 mL) was collected in EDTA-free tubes and centrifugated at 3000 ×g for 10 min to collect the serum.

Hematology: The blood sample was applied for the evaluation of the total count of red blood cells (RBCs), white blood cells (WBCs), and hemoglobin (Hb). The WBCs, RBCs lymphocytes, and platelets were determined using an automated cell counter (HOSPITEX analyzer, Italy) [31].

Serum Biochemical Parameters: A colorimetric approach was utilized to evaluate the levels of aspartate transaminase (AST, 260 001, Spectrum, Cairo, Egypt) and alanine aminotransferase (ALT, 265 001 Spectrum, Cairo, Egypt), while alkaline phosphatase (ALP) was determined in the serum following Zhou et al.[30]. The serum total protein concentrations were determined [32], whereas the quantities of albumin were tested using the technique developed by Zhou et al.[30]. The difference between total protein and albumin calculated the globulin content. The concentration of glucose was measured using the [33] method. The creatinine and urea in serum were determined following [30]. The microplate reader (Infinite M Nano, manufactured by TECAN, Austria) was used in colorimetric measurements.

Antioxidant Enzymes: Malondialdehyde (MDA, cat no. 230001, Spectrum, Cairo, Egypt), the lipid peroxidation marker, was determined following the instructions given using Spectrum kits. The total non-enzymatic antioxidant capacity (TAC) was assessed using the BioDignostic kits following [34]. The measurements were conducted *via* a microplate reader at the respected wavelength (Infinite M Nano, TECAN, Austria).

Histopathological Examination

The liver tissues were picked, preserved in formalin, and processed by an automated processor. An initial phase was fixed and then dehydrated. The fixation was conducted by immersing the tissue for 48 h in 10% formalin, after which the fixation solution was removed using distilled water for 30 min. The tissues were subsequently dehydrated by immersing in elevating levels of alcohol (70, 90, and 100%) for 120, 90, and 90 min, respectively. The dehydration was subsequently cleared using multiple cycles of xylene. The procedure involved submerging the tissue for one h in a solution of 50% xylene & 50% alcohol and then for an additional 1.5 h in pure xylene. The specimens were then saturated with melted paraffin wax, encased, and sealed. Hematoxylin & eosin were used for 4-5 µm paraffin cut sections. Blood circulation disruptions, irritation, degenerations, apoptosis, necrosis & additional histopathological alterations in the tissues were monitored.

Analysis of Inflammatory Cytokines via Real-time PCR

The liver's inflammatory cytokines (TNF-α, IL-6) were evaluated using qPCR. The RNA was isolated using a commercial kit (Thermo Scientific, USA), and then its concentration was estimated by the Quawell Nanodrop instrument (USA). Reverse transcription was utilized to produce cDNA using a RevertAid commercial kit (Thermo Scientific, USA). The qPCR analysis was conducted utilizing an Applied Biosystem StepOnePlus real-time PCR system (USA) in combination with a mix of cDNA and 2X Maxima SYBR Green Master Mix (USA) and primers specific to each gene. As an internal control, the β-actin gene was employed to compute the change in the target genes using reference genes.

Statistical Analysis

The experiments were conducted using an entirely random design (CRD) with triplicates. The triplicate data means were analyzed using one-way ANOVA via Microsoft Excel (v 2108, 2021). The means were compared with the LSD at $P<0.05$.

Results

Essential Oil Content

The results of the GC-MS analysis of the oils are shown for Dhimran *(Ocimum forsskaolii* benth) in *Table 1*, where the eluents are provided in the order of the HP5MS column elution. The primary components identified in the EO of *Ocimum forsskaolii* benth included were endo-Fenchol, tau-cadinol, α-eudesmol, α-terpineol, α-Bulnesene, and β-atlantol. The contents of these volatile compounds were 18.89, 11.6, 8.3, 7.2, 5.2, 10.9%, respectively. Endo-fenchol, tau-cadinol, and β-atlantol volatile compounds

Table 1.GC/MS profile of essential oils detected in Dhimran (Ocimum forsskaolii benth)	
Detected Compounds	**% Area**
endo-Fenchol	18.89±0.9a
tau-cadinol	11.6±1.1b
germacrene B	3.6±0.2e
endo-borneol	2.6±0.3f
isoshyobunone	2.5±0.2f
Bornylene	0.33±0.01j
Germacrene D	0.25±0.03j
Borneol L	0.61±0.01h
delta-Guaiene	0.68±0.02h
α-Pinene	0.5±0.01i
α-eudesmol	8.3±0.6c
α-terpineol	7.2±0.3cd
α-Bulnesene	5.2±0.5d
β-atlantol	10.9±0.8b
β-Pinene	1.5±0.2g
β-Myrcene	0.62±0.02h
β-Thujone	2.2±0.1f
γ-eudesmol	2.4±0.2f
P value	<0.0001
Different Lowercase letters in the same column indicate significant differences at P<0.05	

represented 52% of total VOCs. Medium contents of VOCs represented in germacrene B, endo-borneol, isoshyobunone, β-Thujone, and γ-eudesmol, while other compounds are in low contents

Phenolic Content and Antioxidant Activity

The scavenging power of DEO and DE is shown in *Table 2*. Dhimran extract had the highest total phenolic and flavonoid content, i.e., 53 and 32 mg/g DW, with a relative increase of 28 and 24% of DEO. Accordingly, the antioxidant activity of DE recorded the highest values, 93%, followed by DEO, 89%. DE showed the lowest IC_{50} of 50 µg/mL, followed by DEO.

Table 2. The antioxidant content of dhimran essential oil and ethanolic extract			
Parameters	**Dhimran (*Ocimum forsskaolii* benth)**		**P Value**
	DEO	**DE**	
TPC (mg/g)	42±1.2b	53±3.2a	<0.0001
TFC (mg/g)	27±2.3b	32±1.2a	<0.0001
AA (%)	89±2.2b	93±3.6a	<0.0001
IC50 (µg/mL)	100±1.2a	50±0.9b	<0.0001
Different Lowercase letters in the same raw indicate significant differences at P<0.05. Dhimran essential oil (DEO), Dhimran extract (DE). Total phenolic compounds (TPC); Total flavonoids compound (TFC); Antioxidant activity (AA); Inhibitory concentration (IC)			

Table 3. Phenolic compounds profile of dhimran extract (DE) detected by HPLC	
Phenolic Compounds	**Value in DE (mg/g)**
Gallic	6.5±0.2b
Caffeic	8.6±0.8a
Ferulic	5.9±0.3bc
Sinapic	3.6±0.1c
Synergic	2.5±0.6d
Quercetin	3.8±0.7c
Luteolin	1.8±0.2e
Rutin	2.1±0.3d
Apigenin	2.9±0.3d
Kaempferol	2.2±0.1d
P value	<0.0001
Different Lowercase letters in the same column indicate significant differences at P<0.05	

The polyphenolic profile detected by HPLC is shown in *Table 3*. Phenolic acids and flavonoids were the main constituents of DE. Caffeic acid was the primary phenolic acid (8.6 mg/g), followed by gallic and ferulic acids. The main flavonoid was quercetin (3.8 mg/g), followed by Rutin, Apigenin, and Kaempferol.

Table 4. Antibacterial activity of dhimran essential oil (DEO) and dhimran extract (DE)							
Test Bacteria	**Dhimran Concentration (µg/mL)**						**P Value**
	DE			**DEO**			
	50	**100**	**200**	**50**	**100 Cardamom**	**200**	
S. typhi	11±0.2c	14±0.2b	18±0.1a	9±0.2d	11±0.6c	15±0.3b	<0.0001
C. jejuni	9±0.1d	11±0.5c	15±0.3a	0±0.0	9±0.2d	12±0.2b	<0.0001
L. monocytogenes	11±0.3c	16±0.3b	22±0.4a	9±0.1d	12±0.5c	17±0.3b	<0.0001
S. aureus	15±0.8d	18±0.5c	25±0.7a	12±0.3e	16±0.1d	21±0.5b	<0.0001
Different Lowercase letters in the same raw indicate significant differences at P<0.05. Salmonella typhi, Campylobacter jejuni, Listeria monocytogenes, Staphylococcus aureus. Dhimran essential oil (DEO), Dhimran extract (DE)							

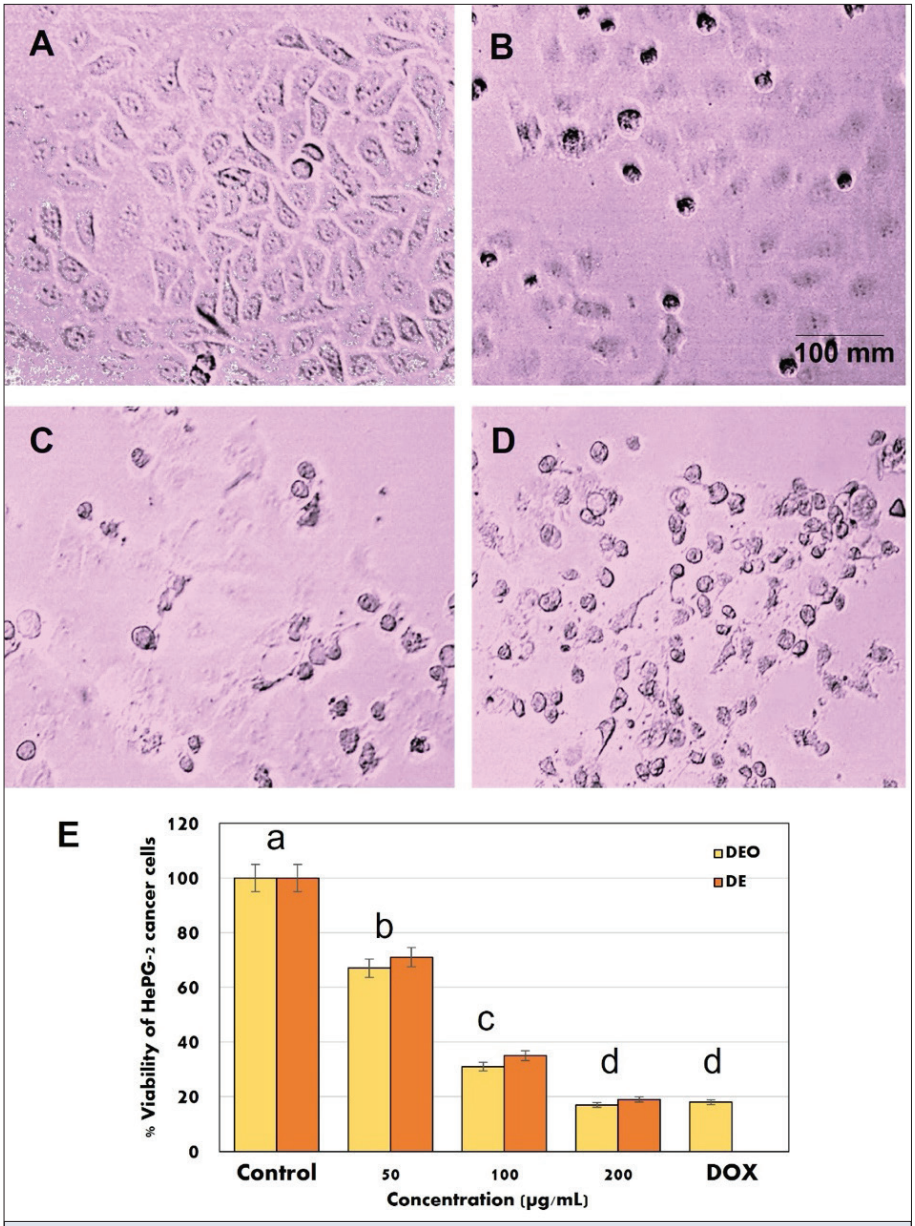

Fig 1. Cytotoxicity of essential oil and ethanolic extract of *Ocimum forsskaolii* benth on the viability of liver cancer cell lines (HePG2) sections stained with H&E (X40), (A) control cancerous cells, (B) *Ocimum forsskaolii* extract (200 µg/mL), (C) *Ocimum forsskaolii* essential oil (200 µg/mL), (D) Doxorubicin (DOX), (E) Histogram of the anticancer effect of *Ocimum forsskaolii* benth essential oil and extract on the viability of HepG2 cell line. Lowercase letters above column indicate signficant differences at P<0.05

Antibacterial Activity of DEO and DE

The summary of the antibacterial activity of DEO and DE against a variety of four bacterial species is presented in *Table 4*. The DEOs and DE were tested for their ability to inhibit gram-positive growth, i.e., *L. monocytogenes* and *S. aureus* and gram-negative bacteria, including *S. typhi* and *C. jejuni*. The ethanolic extract of dhimarn (200 µg/mL) recorded the highest antibacterial activity against *S. aureus* (25 mm) and *S.typhi* (18 mm), with relative increases of 20 and 26% compared to DEO. DE exhibited an inhibition area ranging from 9 to 25 mm against all the pathogens tested. The DEO recorded inhibition zone diameters against the tested bacteria in the 9-21 mm range. DEO (50 µg/mL) has not antibacterial activity against *Campylobacter jejuni*. *C. jejuni* was the most resistant bacteria to DE and DEOs; however, *S. aureus* was the most sensitive to EOs and extract of dhimarn.

Cytotoxic Activity of Dhimarn EOs Against Hepatic Cancer Cell Lines

Fig. 1 shows that each essential oil and ethanolic extract of *Ocimum forsskaolii* benth (dhimarn) has cytotoxicity on human liver cancer cells (HepG2). *Ocimum forsskaolii* benth extract reduces the viability of HepG2 cancer cells

Table 5. *The effect of DEO and DE administration on growth parameters of CCl_4-induced liver cancer*

Parameters	Treatments						P Value
	Control	DE	DEO	CCl_4	DE+CCl_4	DEO+CCl_4	
IBW	30.5±0.2	31.2±0.1	31.6±0.2	30.8±0.5	31±0.2	31±0.6	0.9
FBW	98.5±1.2b	106±2.2a	101.6±3.1ab	73.8±2.1d	90±1.6bc	86±2.2c	<0.0001
BWG	68±1.3c	75±0.9a	70±0.8b	43±1.2e	59±0.9d	55±0.9de	<0.0001
FCR	1.45±0.2b	1.6±0.2a	1.55±0.1ab	1.2±0.2c	1.39±0.1b	1.35±0.2bc	0.0041
SR	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	0.98
ALW	5.1±0.2c	5.3±0.3c	5.2±0.4c	7.9±0.5a	6.1±0.2b	6.0±0.1b	0.0033
ROW	2.5±0.1c	2.56±0.1c	2.7±0.3c	3.8±0.7a	2.9±0.1b	3.1±0.2b	0.00235

Lowercase letters in the same raw indicate significant differences at p<0.05. Absolute liver weight (ALW), Relative organ weight (ROW), survival rate (SR), initial body weight (IBW), final body weight (FBW), and feed conversion ratio (FCR). Dhimran essential oil (DEO), Dhimran extract (DE)

Table 6. *The effect of DEO and DE administration on the hematology of CCl_4 rats-induced liver cancer*

Hematology	Treatments						P Value
	Control	DE	DEO	CCl_4	DE+CCl_4	DEO+CCl_4	
RBCs × 10^6/l	7.5±0.2ab	7.9±0.2a	7.7±0.5ab	4.8±0.2c	6.5±0.2b	6.0±0.2b	0.00021
WBCs × 10^6/l	5.2±0.6c	5.5±0.1c	5.1±0.2c	17.2±1.2a	7.2±1.1b	6.9±0.4b	0.0023
Ht %	43.68±2.2a	42.9±3.1ab	42.0±1.1ab	22.5±2.1c	39.7±0.9b	38.2±1.3b	0.00154
Hb g/dl	14.3±0.3a	14±0.2a	14.2±0.3a	8.2±0.5c	13.1±0.7ab	12.8±0.9b	0.031
Platelets × 10^6/l	820±6.3b	835±3.3a	822±4.1b	350±3.2d	790±2.3c	750±3.6c	0.0042

Different lowercase letters in the same raw indicate significant differences at P<0.05. Dhimran essential oil (DEO), Dhimran extract (DE). Red blood cells (RBCs); White blood cells (WBCs); Hematocrit (Ht); Hemoglobin (Hb)

Table 7. *The effect of DEO and DE administration on the liver markers of CCl_4 rats-induced liver cancer*

Liver Markers	Treatments						P value
	Control	DE	DEO	CCl_4	DE+CCl_4	DEO+CCl_4	
AST	123±2.1a	115±1.1b	113±2.1b	85±1.1d	105±0.9c	100±0.0c	0.0014
ALP	355±3.6a	320±2.1c	318±3.1c	260±2.1d	340±3.3b	338±3.2b	0.0051
ALT	165±1.2a	152±2.3b	150±1.2b	120±1.3d	144±2.1c	140±1.1c	0.0085
LDH	789±2.2d	820±6.1c	817±6.2c	989±7.0a	856±8.3b	850±8.8b	0.0041
TP	175±1.1b	189±3.2a	180±1.2ab	122±1.1d	160±1.1c	156±2.1c	0.0011

Lowercase letters in the same raw indicate significant differences at p<0.05. Dhimran essential oil (DEO), Dhimran extract (DE)

by 86%. *Ocimum forsskaolii* benth EO inhibited 82% of cancerous cell viability; all results compared with Doxorubicin reduced cancerous cell proliferation by 85%. The results correlated with microscopic images. On the other hand, the IC_{50} of treatments inversely correlated with the inhibition percentage, where the IC_{50} of DE was lowest after DOX (20 µg/mL), followed by DEO (31 µg/mL).

In vivo Experiment of Hepatoprotective Impact of DEO and DE on CCl_4 Toxicity

Growth Parameters: Clinical poisoning manifestations were observed in the CCl_4 mice, such as salivation, digging, trembling, squirming, and convulsions; however, there were no mortalities. Compared with the control group, an increase in relative liver weights (ALW and ROW) and considerable reduction in weight gain and FCR were observed in CCl_4 mice. These parameters were significantly reduced when treating the mice with DEO or DE with a preference for DE. No significant differences were observed in the single application of DEO, DE, and control *(Table 5)*.

Hematology: The oral administration of CCl_4 to mice resulted in notable reductions in levels of red blood cells (RBCs), hematocrit (Ht), hemoglobin (Hb), and platelets; however, a raise in white blood cells (WBCs). The single application of DEO or DE did not affect the hematological parameters (P>0.05). However, when the combined application DEO+CCl_4 or DE+CCl_4 significantly P<0.05

restored all levels to the control levels compared to the CCl_4 group *(Table 6)*.

Liver Enzymes: The liver (ALP, AST, ALT, and LDH) enzymes are of interest as potential hepatotoxicity biomarkers. The administration of CCL_4 in mice lowered total protein content; however, it raised the activity of AST, ALT, ALP, and LDH in mice liver homogenate compared to the control ($P<0.05$). The combined oral application of DEO+CCl_4 or DE+CCl_4 significantly $P<0.05$ reduced the activity of liver enzymes and increased the total protein content. The single application of DE or DEO substantially altered the studied parameters *(Table 7)*.

Lipid and Protein Oxidation Markers: A notable increase was observed in the concentrations of lipid hydroperoxides (LOOH) after the administration of CCl_4 compared to the control group. However, mice that were administered DEO+CCl_4 or DE+ CCl_4 exhibited a substantial reduction in the concentrations of LOOH in comparison to the CCl_4-treated group *(Fig. 2-A)*. Furthermore, the hepatic levels of protein oxidation indicator, advanced oxidized protein products (AOPP), were significantly elevated in the CCl_4-intoxicated group. The oral application of DEO or DE reduced the high concentration of AOPP. The levels of lipid peroxidation and protein oxidation in the DEO-mice or DE-mice differed significantly compared to the control group *(Fig. 2-B)*.

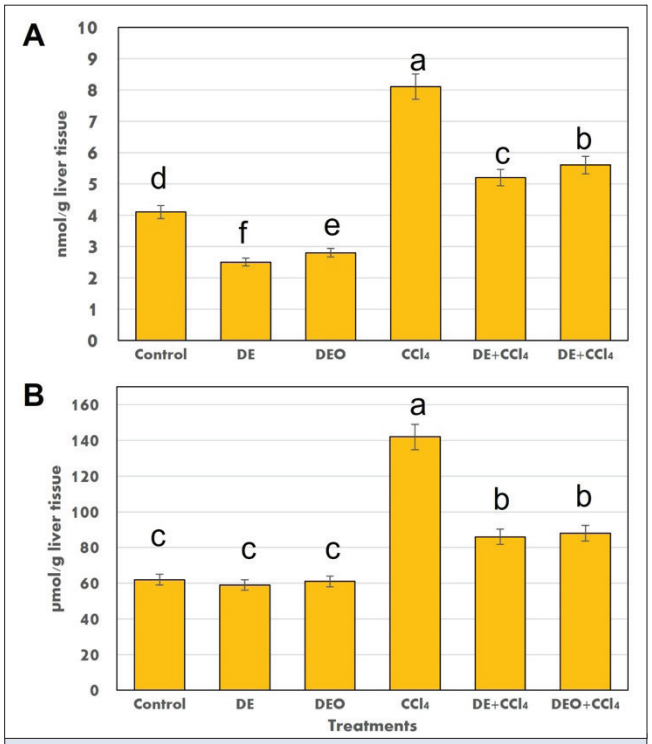

Fig 2. The effect of DEO and DE on mitigating oxidative stress induced by hepatocytes CCl_4 administration, (A) Lipid hydroperoxide (LOOH), (B) Advanced oxidized protein product (AOPP). The significant differences between treatments are indicated by letters above columns ($P<0.05$)

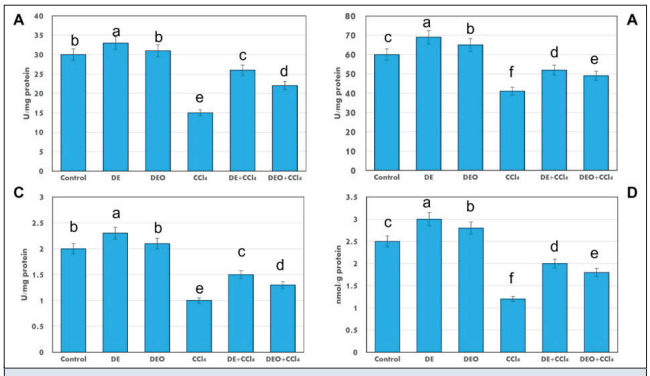

Fig 3. The effect of DEO or DE on enhancing the antioxidant system defense in hepatocytes rats, (A) SOD activity; (B) Catalase activity; (C) GPx activity; (D) GSH activity. The significant differences between treatments are indicated by letters above columns ($P<0.05$). Dhimran essential oil (DEO), Dhimran extract (DE)

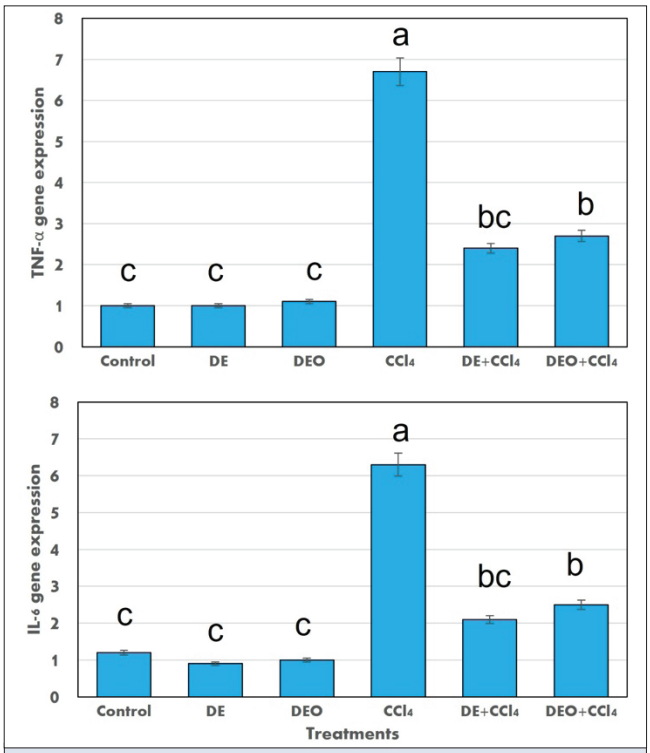

Fig 4. Relative expression of *IL-6* and TNF-α pro-inflammatory cytokines in mice liver tissues of different groups. The significant differences between treatments are indicated by letters above columns ($P<0.05$). Dhimran essential oil (DEO), Dhimran extract (DE)

Antioxidant Status: The liver homogenates of CCl_4-mice show a significant decrease in the non-enzymatic defense, glutathione (GSH), as well as lower activity of the antioxidant system (SOD, CAT, and GPx) when compared to the control group. However, there was a notable increase in these parameters in the mice treated with combined application of DE+CCL_4 or DEO and CCl_4, compared to CCL_4. The indices are substantially enhanced ($P<0.05$) by the sole application of DEO or DE *(Fig. 3)*.

Inflammatory Cytokines: The liver tissue of mice administered CCl_4 exhibited notable elevations in the

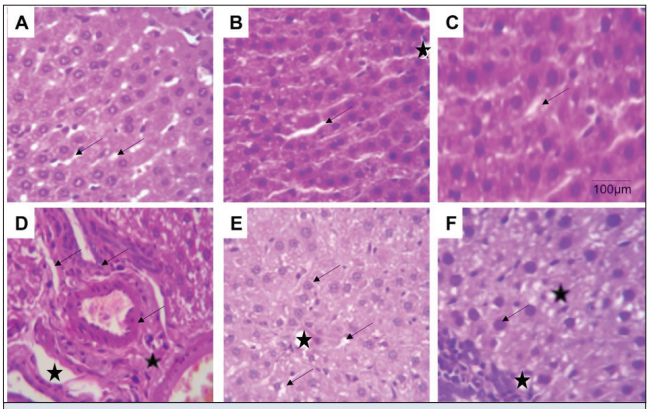

Fig 5. Photographs liver sections stained by H&E (X40) showing a) normal hepatic central vein and hepatic cords in control group; b) DE group, typical histological structure of hepatic lobule; c) DEO group showed standard histological structure; d) The CCl_4 group showed perivascular collagen fiber deposits adjacent to severe acute cell swelling; e) DE+ CCl_4 group mild peribiliary lymphocytic infiltrations adjacent and recovery of normal tissues; f) DEO+ CCl_4 group mild peribiliary lymphocytic infiltrations adjacent and recovery of normal tissues

mRNA levels of TNF-α and IL-6-related inflammation, as determined by qPCR, compared to the control group. When mice were orally pretreated with DEO or DE and subsequently intoxicated with CCL_4, the examined inflammation cytokines exhibited significant ($P<0.05$) downregulation *(Fig. 4)*.

Histological Studies

Liver section tissue in control, DE, and DEO groups showed maintained liver cords, portal triad's structures, biliary system, vascular tributaries, sinusoids, Von Kuepfer's cells, and supporting stroma *(Fig. 5-A,B,C)*. The liver section of 1/10 CCl_4 LD_{50} treated groups showed portal biliary proliferative reactions. The portal blood vessels appear moderately to markedly dilate with occasional portal edema and infiltration of round cells (lymph-plasmacytes). The hepatic sinusoids are mild to moderately dilated, sometimes with atrophy of the surrounding hepatocytes *(Fig. 5-D)*. Hepatic Sections from 1/10 CCl_4 LD_{50} co-treated with DEO or DE revealed mild to moderate vascular dilatations, round cell aggregations, and recovery to the normal liver tissues *(Fig. 5-E,F)*.

Discussion

Plant compounds are commonly used as alternative medicinal agents due to their reduced adverse side effects. The global market has witnessed a surge in the demand for research about natural products owing to their challenging therapeutic attributes [35]. Furthermore, research has indicated that the escalating demand is significantly influenced by the therapeutic chemical composition and biological activity of natural products, such as essential oils (EOs), extracts, and other plant-based products, that possess surplus medicinal values [36].

In this study, the essential oils of *Ocimum forsskaolii* benth was analysed. Similiarly, in a study by Bader et al.[14], the essential oil of Ocimum forsskaolii benth was found to contain methyl eugenol, eugenol, linalool, germacrene D, and β-caryophyllene as its primary constituents. These compounds comprised between 2.57% and 55.65% of the oil.

Also, Elansary and Mahmoud [37] found the main compounds in the *O. basilicum* oil were methyl cinnamate (43.8%) and chavicol methyl ether (39.1%).

The considerable content of EOs in basil cultivars possess various biological activities where the obtained results were consistent with other findings Nguyen et al.[38] stated that the volatile oil extracted from *Ocimum basilicum* by distillation steam and a Clevenger-type device, that have antioxidant activity determined by DPPH and linoleic acid peroxidation. The findings demonstrated that essential oils have antioxidant characteristics and revealed that the basil leaf extract exhibited a substantial antioxidant capacity, as evidenced by its IC50 of 285.36 µg/mL.

Furthermore, Tshilanda et al.[39] determined the anti-oxidant potential of the basil plant using the DPPH free radical scavenging activity method. The essential oil had antioxidant properties, with an IC_{50} of 1180 µg/mL. The methanolic and ethyl acetate extracts showed higher antioxidant activity than essential oil, with IC_{50} values of 25 and 85 µg/mL, respectively. The essential oil was less active than methanol and ethyl acetate extracts regarding IC_{50} values. The most active extract was methanol crude extract. The ability of non-polar extracts to scavenge radicals was minimal; hence, IC_{50} values could not be determined. Also, Qasem et al.[6] estimated the antioxidant properties of basil *(Ocimum basilicum* L.) extracts that inhibit DPPH radicals with IC_{50} 20 µg/mL; this activity because of the considerable content of the total flavonoids from 40 mg/g and total phenolic of 65 mg/g.

The antimicrobial activity of Dhimran essential oil affects the development of three different kinds of bacteria. According to the survey findings, essential oils have a strong antibacterial effect on all Gram (+) and Gram (-) bacteria strains and the fungus *C. albicans*. The capacity of an essential oil constituent to permeate the cell walls of a bacteria or fungus is directly correlated with how soluble they are in water. Therefore, the solubility of essential oils in the phospholipid bilayer of cell membranes accounts for their antibacterial activity [40]. Consistent with the current study, the essential oil showed superior antibacterial activity against gram-positive bacteria *(S. aureus)* and moderate activity against gram-negative bacteria *(E. coli)*, as evaluated by the agar diffusion method. The growth of bacteria was only faintly suppressed by essential oil at low concentrations (5 µL). Numerous literature studies

have shown that several *Ocimum basilicum* essential oil components have antibacterial properties [41].

The essential oils and extract of Dhimran possess anti-cancer activity against liver cancer cell lines, as the obtained results stated; in this regard, Eid et al.[15] indicated basil essential oil demonstrated potent anticancer activity against Hep3B (IC_{50} 56.23 µg/mL) and MCF-7 (80.35 µg/mL) compared to Doxorubicin. The research indicates that the extract of *Ocimum basilicum* has an anti-proliferative effect on liver cancer cells due to the presence of phytochemicals, specifically phenolic compounds [42].

This research investigates the impact of CCl_4 exposure on protein oxidation products and lipid peroxidation, including AOPP and LOOH [43]. The observed elevation in free radical concentrations and compromised antioxidant defenses indicate that CCl_4 may induce cellular harm by inhibiting membrane mobility.

Liver fibrosis, distinguished by the accumulation of collagen in the liver, is affected by the detected elevation in hydroxyproline (HYP), the principal constituent of collagen [44].

Glutathione is a vital constituent of the antioxidant defense system, aiding in maintaining cellular redox equilibrium and protecting against free radicals [45]. Increased lipid and protein oxidation (LPO) levels, which indicate heightened ROS production, may account for the observed decrease in GSH levels and the activities of GPx and GR in rats treated with CCl_4 [5]. It is believed that enzymatic antioxidants (SOD and CAT) serve as the initial line of defense against the detrimental impacts of reactive oxygen species (ROS) produced by CCl_4 on biological macromolecules [46].

The liver enzymes (ALT, AST, ALP, and LDH) significantly changed in CCl_4 mice because of the disruption of cellular membrane integrity caused by LPO [5]. This disruption permits the release of cytoplasmic enzymes into the bloodstream after hepatocellular injury.

The present investigation examines the hepatic impacts of CCl_4, explicitly focusing on inflammation and the expression of TNF-α and IL-6 genes. Additionally, the function of reactive oxygen species (ROS) in producing pro-inflammatory cytokines such as IL-12, INF-α, and TNF-α is investigated. Reactive oxygen species (ROS) have a solid ability to stimulate the NF-kB transcription factor, an elemental component of inflammation and innate immunity [47]. Elevated levels of IL-6 and TNF-α correlate with increased expression of pro-apoptotic genes IL-1β, IL-10, NF-kβ, Bax, and P53 [48].

Depletion of hepatic proteins signifies a breach in the integrity of the cell membrane, which permits the entry of toxicants into the cells [48]. Reducing hepatic protein levels can impact genetics by stimulating hepatic cell growth through alterations in the cell cycle.

CCl_4 accumulates in mice's liver tissues, which induces a surge in the production of free radicals, oxidative stress, and apoptosis [49]. It was demonstrated that *Ocimum gratissimum* (OG), *O. basilicum* (OB), and *O. tenuiflorum* (OT) extracts possess more anti-inflammatory characteristics in their ability to scavenge nitric oxide and inhibit lipoxygenase [50].

In this finding, the oral administration of DEO and DE decreased LPO and increased antioxidant status due to the oil's diverse phenolic components and natural products [51]. DEO's decreased radical scavenging activity was almost certainly attributable to its principal component, estragole [20]. DEO and DE supplementation alleviated the fluctuation of the antioxidant defense system and liver biomarkers induced by CCl_4 [52]. Due to its high concentration of phenolic compounds, including flavonoids and phenolic acids, basil possesses redox properties and can scavenge free radicals [53]. With a substantial reduction in hydroxyproline deposition in hepatocytes, basil is an effective treatment for hepatic fibrosis [54]. The study observed fibrotic changes in the liver and found that it stimulates liver regeneration in mice with fibrosis, which was alleviated by basil [55]. Because of its strong ability to remove ROS and reduce inflammation, the essential oil of basil leaves might mitigate the cytotoxic impact of CCl_4 when utilized as a phytochemical drug.

CCl_4-induced oxidative stress disrupted liver function and antioxidant status biomarkers in mice liver tissue. A pre-treatment with DEO or DE restored the efficiency of enzymes and biomarkers. DEO and DE demonstrated substantial antioxidant, antibacterial, and anticancer effects attributed to their volatile molecules and phenolic constituents, which are scavengers for free radicals. Furthermore, the hepatotoxicity generated by CCl_4 in mice, including cell cycle arrest, inflammation, oxidative stress, and DNA damage, can be alleviated by DEO and DE's anti-inflammatory and antioxidant capabilities. Therefore, Dhimran essential oil or ethanolic extract is a commonly accessible and affordable botanical treatment with significant antioxidant activity.

Declarations

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

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The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

• Copyright

The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veteriner Fakultesi Dergisi
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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 5, Year: 2024

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

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ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (E-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

2- The official language of our journal is English.

3- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and other files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

4- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

5- Authors should know and take into account the "Generative Artificial Intelligence (AI)" and other matters listed in the **"Ethical Principles and Publication Policy"** section regarding scientific research and authors.

6- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

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.

8- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

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.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
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9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

11- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

12- The editorship may request the language editing of the manuscript submitted to the journal. If the article is accepted, it will not be published without language editing. Before publication, a declaration and/or certificate stating that proofreading is done by a registered company will be requested from the corresponding author.

13- No fee is charged at any stage in Kafkas Üniversitesi Veteriner Fakültesi Dergisi (No APC/APF)

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover Letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title Page

• Title, Running Title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

- Declarations

• Availability of Data and Materials

• Acknowledgements

• Funding Support

• Competing Interests

• Generative Artificial Intelligence (AI)

• Authors' Contributions

Further Considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided