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Contact (Editor in Chief)
Mustafa Sinan AKTAŞ
Department of Internal Medicine, Atatürk University, Faculty of Veterinary Medicine, Erzurum, Türkiye
vetdergisi@atauni.edu.tr
https://dergipark.org.tr/en/pub/vetsci

Contact (Publisher)
Atatürk University
Atatürk University, Erzurum, Turkey
ataunijournals@atauni.edu.tr
https://bilimseldergiler.atauni.edu.tr
+90 442 231 15 16

Veterinary Sciences *and* Practices

ABOUT

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

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

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

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

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

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

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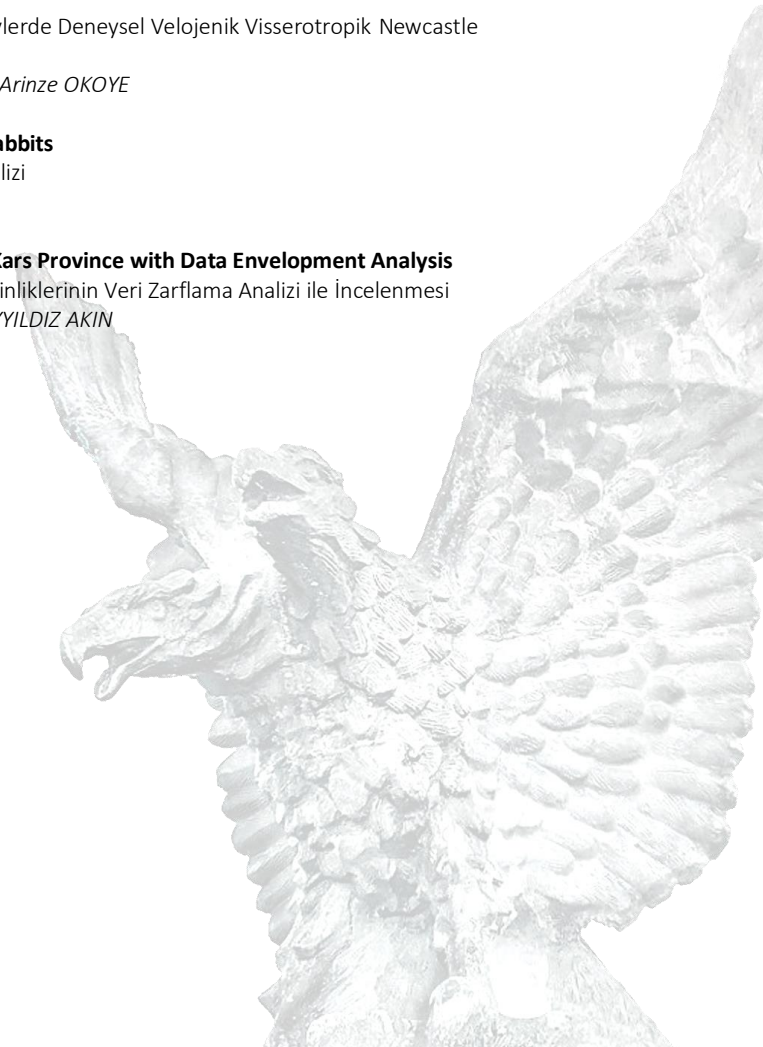
Veterinary Sciences
and Practices

CONTENTS / İÇİNDEKİLER

RESEARCH ARTICLES / ARAŞTIRMA MAKALELERİ



ATATÜRK ÜNİVERSİTESİ YAYINLARI
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Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1471527

Knowledge and Practices of Poultry Farmers Contributing to Antimicrobial Resistance in Nsukka

Nijerya, Enugu Eyaleti, Nsukka'daki Tavuk Yetiştiricilerinin Antimikrobiyal Dirence İlişkin Bilgi ve Uygulamaları

Gladys Chinelo Ezeh[1,2]
Akwoba Joseph Ogugua[1]
Michael Olatunji Olaolu[2]
John Anaelom Nwanta[1]

[1]University of Nigeria, Enugu State, Akwoba Joseph Department of Veterinary Public Health and Preventive Medicine, Nsukka, Nigeria
[2]Department of Agriculture, Faculty of Agriculture, Alex Ekwueme Federal University, Ndufu Alike, Ebonyi State, Nigeria

ABSTRACT

Antimicrobial use in animal husbandry has been ascribed to antimicrobial resistance (AMR) gene selection and build-up in treated animals' microbiota. This ends up in the food chain and contributes immensely to drug resistance in the society. Studies on risk factors for antimicrobial resistance in poultry can be useful in providing data and designing appropriate control measures. This study therefore assessed the knowledge and practices affecting AMR in poultry farms in Nsukka, Enugu State, Nigeria. A semi-structured and pre-tested questionnaire was administered to 44 poultry farmers in the study area. Among the farmers, 90.91% were aware that excessive antimicrobial use contributes to the emergence of antimicrobial resistance. More than 70% of the farms lacked basic hygiene and biosecurity facilities/measures. Sixty percent of the farmers buried their dead birds, all (100%) packaged their dung for subsequent land disposal, and 50% dumped expired, unused/used drug packets in the nearest bush. About 65, 100, and 90% of antimicrobial usage were for growth promotion, prophylactic purposes, and therapeutic purposes, respectively. Finally, only 18.18% observed withdrawal periods before disposal of their products. The study found that the farmers used non-therapeutic antimicrobials as a "simple fix" or to compensate for poor management practices. There is need to further educate the farmers on the contributions of their activities to drug resistance in the society.

Keywords: Antibiotic use, antimicrobial resistance, knowledge, poultry farm, practices

ÖZ

Hayvancılıkta antimikrobiyal kullanımı, antimikrobiyal direnç (AMR) gen seçimine ve tedavi edilen hayvanların mikrobiyotasında birikime neden olmaktadır. Bu durum gıda zincirinde son bulmakta ve toplumdaki ilaç direncine büyük ölçüde katkıda bulunmaktadır. Kanatlı hayvanlarda antimikrobiyal direnç için risk faktörleri üzerine yapılan çalışmalar, veri sağlama ve uygun kontrol önlemlerinin tasarlanması açısından faydalı olabilir. Bu çalışmada Nijerya'nın Enugu Eyaleti, Nsukka'daki tavuk çiftliklerinde AMR'yi etkileyen bilgi ve uygulamalar değerlendirilmiştir. Çalışma alanındaki 44 tavuk yetiştiricisine yarı yapılandırılmış ve önceden test edilmiş bir anket uygulanmıştır. Yetiştiricilerin %90,91'i aşırı antimikrobiyal kullanımının antimikrobiyal direncin oluşumuna katkıda bulunduğunun farkındaydı. Çiftliklerin %70'inden fazlası temel hijyen ve biyogüvenlik tesislerinden/önlemlerinden yoksundu. Yetiştiricilerin %60'ı ölü tavukları gömmüş, tamamı (%100) gübrelerini daha sonra araziye atmak üzere paketlemiş ve %50'si son kullanma tarihi geçmiş, kullanılmamış/kullanılmış ilaç paketlerini en yakın çalılık araziye atmıştır. Antimikrobiyal kullanımının yaklaşık %65, %100 ve %90'ı sırasıyla büyümeyi teşvik etme, profilaktik amaçlar ve tedavi amaçlıydı. Son olarak, yetiştiricilerin sadece %18,18'i ürünlerini imha etmeden önce atılım sürelerine dikkat etmiştir. Çalışma, yetiştiricilerin "basit bir çözüm" olarak veya kötü yönetim uygulamalarını telafi etmek için non-terapotik antimikrobiyaller kullandığını ortaya koymuştur. Toplumdaki ilaç direncine katkılarının farkında olmaları için yetiştiricilerin daha fazla eğitilmesi gerekmektedir.

Anahtar Kelimeler: Antibiyotik kullanımı, antimikrobiyal direnç, bilgi, tavuk çiftliği, uygulamalar

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Corresponding author/Sorumlu Yazar:
Akwoba Joseph OGUGUA
E-mail: ogugua.akwoba@unn.edu.ng

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INTRODUCTION

Poultry meat and eggs provide a substantial portion of the dietary needs of the world's rapidly growing population and remain the world's foremost sources of animal protein.[1] Over the last 35 years, global poultry meat and egg production, as well as sales of poultry products, have witnessed significant increase.[2,3] In Nigeria, there are about 180 million poultry birds generating 650,000 metric tons of eggs and 300,000 metric tons of meat every year, with approximately 13 million families engaging in poultry production.[4] As a result, the poultry sector in Nigeria is an important source of animal protein, accounting for about 10% of agricultural gross domestic product (GDP) and 3.1% of national GDP.[4] In addition, poultry manure has become an inseparable part of agriculture in improving soil fertility and optimum crop yield.[5] Increased demand for poultry products therefore, has resulted in many poultry farmers resorting to unwholesome use of antimicrobials.[6] Presently, antimicrobials are used for both curative and prophylactic purposes.[7] This use of antimicrobials for optimal productivity has resulted to drug residues in the tissues and organs of the treated animals, even crops[8] which eventually reach the human population via the food chain.[9] Exposure to drug residues through poultry products has been connected to allergic reactions, changes in intestinal microbiota, and, eventually, the emergence of antimicrobial resistance (AMR) among the consumers.[10]

Antimicrobial resistance naturally occurs when microorganisms are exposed to antimicrobials which exerts selection pressure on them thereby resulting to a loss of sensitivity to such drugs.[11, 12] It is a key threat to both animal and human health and has major ramifications for public health due to the evolution of multi-drug-resistant pathogens that have become a major source of concern to veterinarians, physicians, and food microbiologists.[13] The increased use of antimicrobial agents as prophylactic and therapeutic agents has been linked to an increase in the prevalence of antimicrobial-resistant Enterococcus species,[14] as well as other resistant bacteria organisms. Humans usually are exposed to these resistant pathogens by handling and consuming contaminated products. Once acquired, such resistant bacteria populations invade intestinal tract and propagate the genes responsible for antibiotic resistance to different bacteria in the endogenous microflora, making effective bacterial infection treatment more difficult.[14]

In Nigeria, the administration of antibiotic formulations with multivitamins and minerals is common in the poultry business.[4] The country's challenges in effectively reducing excessive antimicrobial usage have been attributed to several factors, including the unrestricted sale of antibiotics without prescription, inappropriate or sub-therapeutic use in food animals, proliferation of unregulated pharmacies, and dearth of information regarding antimicrobial resistance and poor knowledge of proper antimicrobial usage.[15]

Several studies have documented the perceptions of antimicrobial resistance risk among poultry farmers in some parts of the world.[16,17] and likewise in Nigeria.[4,15] However, there is a dearth of information on the above subject matter among poultry farmers in Nsukka, Enugu State, Nigeria. This study was therefore designed to determine the knowledge and practices concerning antimicrobial resistance among poultry farmers in Nsukka, Enugu State, Nigeria.

MATERIALS AND METHODS

Study Area

The study was conducted in Nsukka, Enugu State, Nigeria. The town is geographically located at coordinates 6°27'9.60''N 7°30'37.20''E.[18] In Nsukka town, there is a sizeable number of poultry farmers who rear commercial birds most of whom belong to the local poultry association (The Nsukka Poultry Farmers Association). The town, as well, harbors a big poultry market, patronized by numerous neighboring towns and the University of Nigeria, Nsukka community.

Study Design, Sample Size Determination, and Sampling of Farms

This cross-sectional study undertook a survey among poultry farmers in Nsukka, Enugu State, Nigeria, between August and December 2019. A comprehensive list of poultry farms was obtained from the chairman of the Nsukka Poultry Farmers Association. With the list of the farms obtained from the chairman as sampling frame, simple random sampling by balloting was conducted targeting 10% of the farms. The managers/representatives of each chosen farm were contacted via mobile phones. The study was thoroughly explained to the respondents and with their anonymity and confidentiality assured, oral informed consent was obtained. Subsequently, an interviewer administered questionnaire was used to elicit information from the respondents on a visit to each farm. Only farmers that belonged to the association and gave consent were recruited for the study. Poultry farmers that were not members of the famers association and those that did not give consent were excluded. In all, forty-four (44) poultry farms and farmer's/farm managers were surveyed in this study.

The Questionnaire Survey

A semi-structured and pretested questionnaire was used to obtain data on demographical characteristics, knowledge of appropriate and the consequences of inappropriate use of antibiotic in food-producing animals; the types and conditions of antimicrobial usage, as well as husbandry and biosecurity practices in each poultry farm. The questionnaire was translated to the local dialect for the benefit of those not fluent in English language and administered to each respondent.

Scoring of Responses

There were four questions in the knowledge survey, and three (75%) correct responses were considered as good, while less scores were considered poor knowledge.

In the practices section, out of the fifteen questions on biosecurity measures subsection, scores of ≥10 or less were regarded as "good" or "poor", respectively. For the class of antimicrobial agent used, giving ≥4 or <4 correct answers out of eight questions were regarded as good or poor scores, respectively. Furthermore, ≥ 3 or < 3 correct responses out of five questions regarding conditions for antimicrobial usage were scored good or poor, respectively. Correct responses ≥2 or < 2 out of three questions was regarded as good or poor methods of disposal of used or expired drug packets respectively. Correct responses of ≥ 4 or < 4 out of five questions were adjudged as good or bad practices with regards to the disposal of dead birds, respectively.

Approval for this study was obtained from the ethical committee of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Nigeria, Nsukka (date: April 17, 2019, reference no: VPHPM/UNN/23/202).

Data Presentation and Statistical Analysis

Data from the survey were analyzed descriptively and presented in tables. Chi-square statistic was used to test for significant association between the sociodemographic characteristics of the respondents, and knowledge as well as practices concerning antimicrobial resistance (KAMR) in the farms visited. Furthermore, the factor score analysis was used as a part of the adjusted multivariate logistic regression analysis to determine the association with key themes regarding the respondents' demographics. Results were expressed as odds ratios (ORs) at 95% confidence intervals (95% CIs), and $P < .005$ used as the threshold for statistical significance. All statistical analysis was done using SPSS version 25 (IBM SPSS Corp., Armonk, NY, USA).

RESULTS

This study was undertaken to gain insight into the challenges, possible control measures given that at the moment there are no programs in place to combat drug resistance in domestic animals in Nigeria. The study was therefore conducted in 44 poultry farms which represent 10% of the farms in the study area.

Demographic Characteristics of the Respondents (poultry farmers) in Nsukka Area

The demographic characteristics of the respondents as shown in figure 1, showed that most of the farmers (72.73%) were males, and attained tertiary education level (81.82%). Approximately 41% of the farmers had 10 years' experience in poultry production, with 59.09% and 31.82% rearing layers and broilers, respectively. As it pertains to the sizes of the farms, 4.55% and 36.36% of the farms had more than 10,000 and fewer than 500 birds, respectively. All the farms (100%) engaged in intensive system of production, where 86.36% and 13.64% were of the deep litter and battery cage management systems, respectively.

Knowledge of Antimicrobial Resistance and Conditions of Antimicrobial Use Among Farms Sampled in Nsukka Area

Figure 2, depicts the farmers' knowledge of antimicrobial resistance. About 90.91% of them were aware that imprudent use of antimicrobial agents and non-observance of withdrawal period could result to antimicrobial resistance. Figure 3, summarizes the antimicrobial usage conditions observed in the poultry farms visited. While most of the farms (72.7%) sourced drugs from the veterinary pharmacies, in most cases (100 and 95.45%), the drugs were used for prophylaxis and therapeutic purposes, respectively. Moreover, 72.73 and 63.64% of the farmers consulted veterinarians for diagnosis and subsequent prescription of drugs, respectively. However, only 18.18% of the farmers reported observing the withdrawal period.

Biosecurity Measures and Waste Disposal Methods Used in the Farms Sampled in the Study Area

The biosecurity measures adopted by the various farms are presented in figure 4. All the farms (100%) visited were fenced and had net. Of the farms sampled, 18.20, 9.10, 27.30, 18.20, 54.50, and 95.90% observed the all-in-all-out principle, quarantine measures, wearing protective clothing, hand washing facilities, use of foot dips, and regular vaccination, respectively. Figure 5, depicts the waste disposal systems available in the farms.

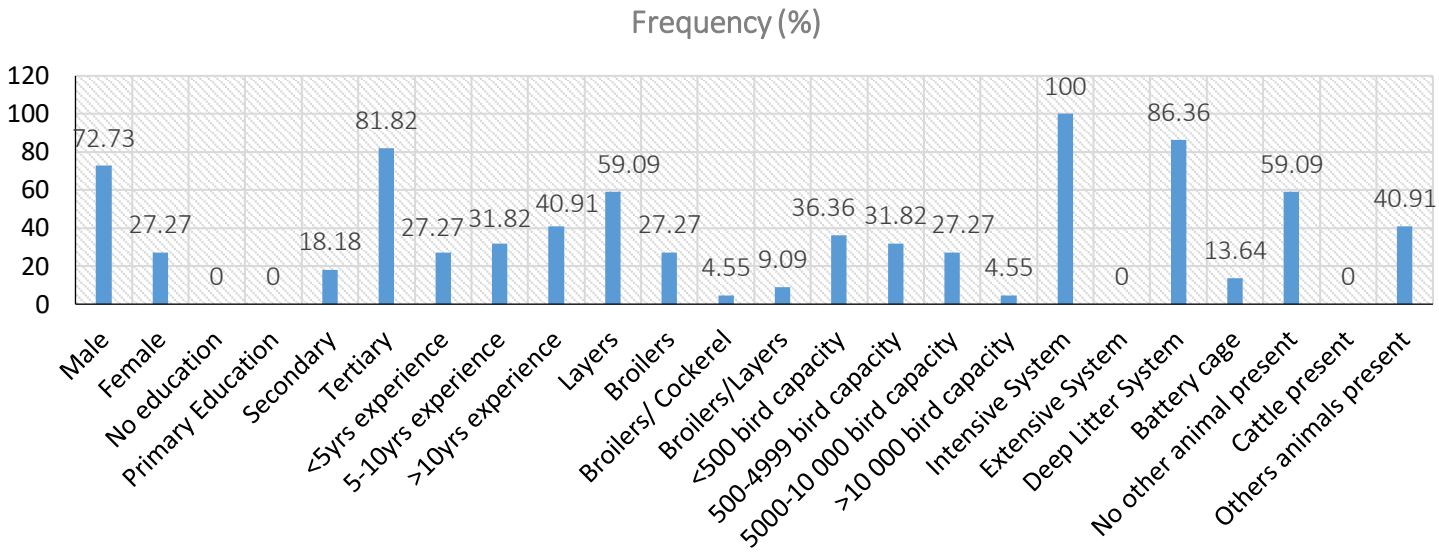

Figure 1: Demographic characteristics of poultry farmers within Nsukka

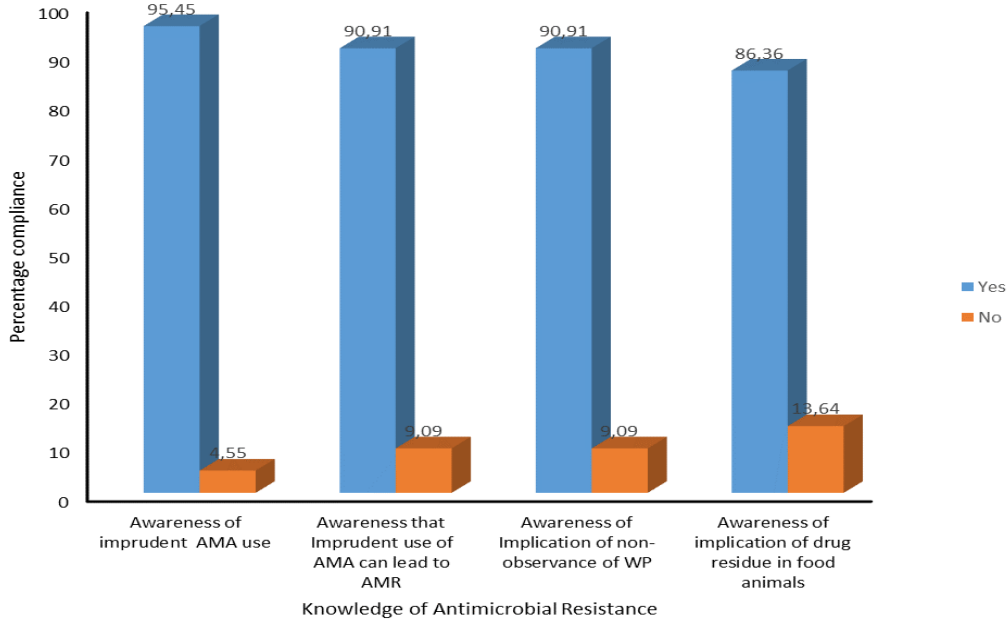

Figure 2: Knowledge of antimicrobial resistance among poultry farmers in the study area.
AMA: Antimicrobial Agents; WP: Withdrawal Period; AMR: Antimicrobial Resistance.

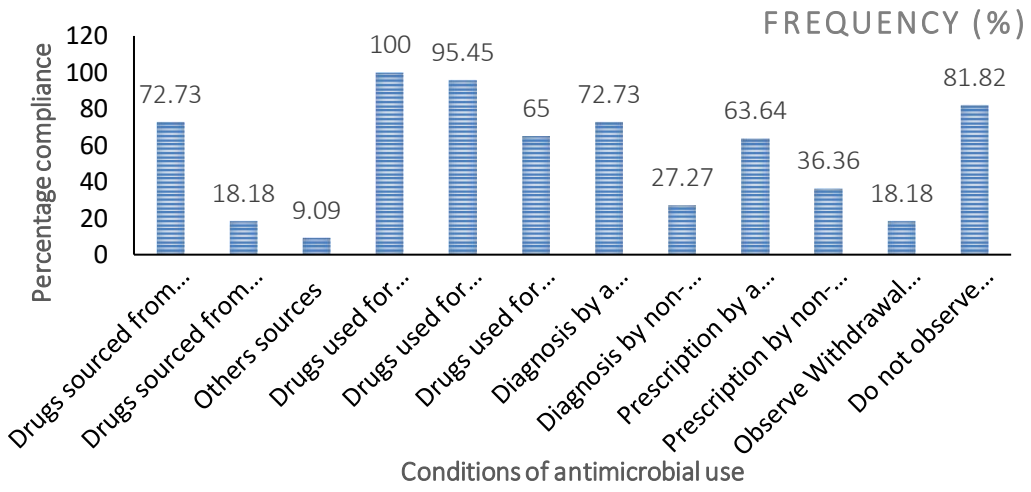

Figure 3: Conditions of antimicrobial use in farms sampled in Nsukka town.

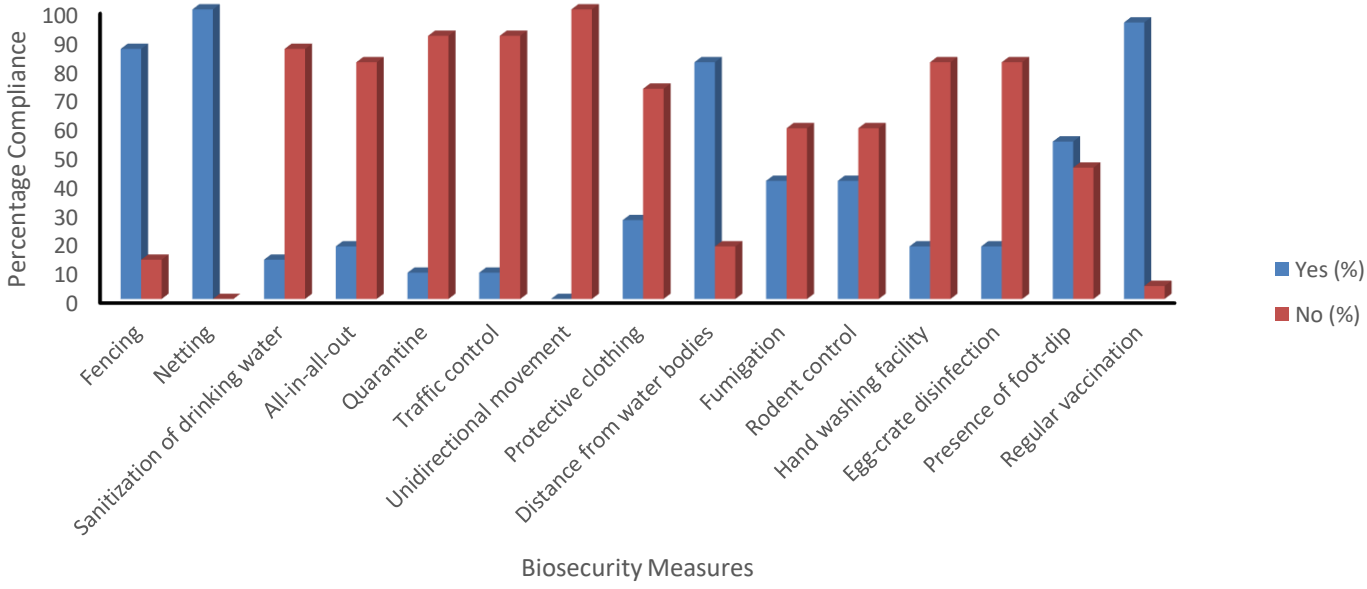

Figure 4: Biosecurity measures in the farms sampled in Nsukka town.

The respondents (100%) reported not selling or eating dead birds which were disposed of in the bush, fed to the dog, buried, or burned by 27, 13, 64, 68, and 54.55% of the farms, respectively. All (100%) the farmers adopted composting as a means of manure disposal, while 50% of them dumped expired drugs and used drug packets into the bush.

Types and Frequency of Antimicrobials Used in Poultry Farm in the Study Area

The types and frequency of antimicrobial usage in the study area is presented in figure 6. Different antimicrobial agents belonging to eight classes of antimicrobial drugs were used by the farmers. The most frequently used classes of antimicrobials were tetracyclines (100%), macrolides (100%), aminoglycosides (94%), and penicillin (63%), while polymyxin (13%) and chloramphenicol (0%) were rarely used.

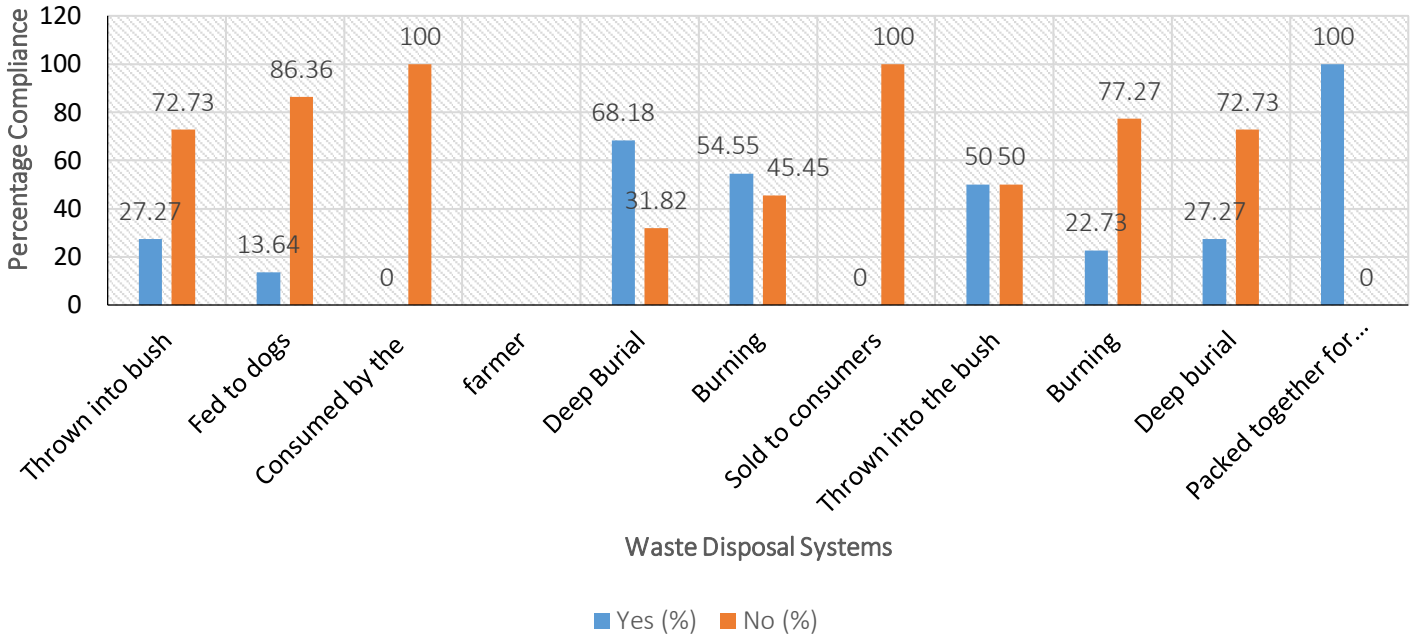

Figure 5: Waste disposal systems among farms sampled in Nsukka Area

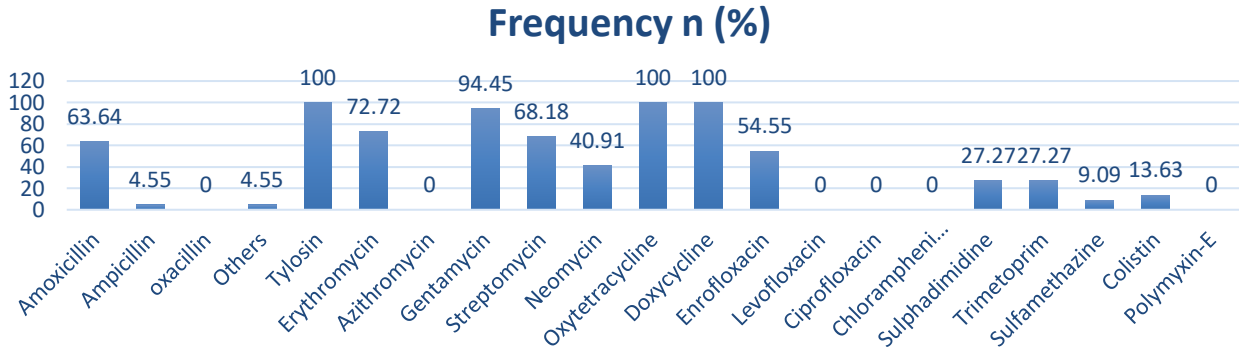

Figure 6: Types and frequency of antimicrobial usage in the farms sampled in Nsukka area.

Factors Associated with the Knowledge of Antimicrobial Resistance Among Poultry Farmers in Nsukka Area

Tables 1 to 5, depict the levels of association between the demographic characteristics of the farmers and their knowledge as well as practices as it concerns antimicrobial resistance. Knowledge of antimicrobial resistance among farmers showed statistical significant association with their level of education (p =0.001), gender (p=0.004), farm size (p =0.048), and the type of bird reared (p =0.001). Statistical significant associations was also observed between practices and: farming experience (p =0.022), farm size (p = 0.031), knowledge of antimicrobial resistance (p = 0.004), and the type of bird reared (p = 0.051), respectively.

Table 6 presents the findings of the adjusted logistic regression analysis of the respondents' demographic characteristics and their level of knowledge, and practices. Most importantly, the findings revealed that farmers' educational level was strongly associated with their knowledge of antimicrobial resistance (KAMR) (OR = 70.210, CI = 4.646-161.005, p = 0.002).

Table 1. Bivariate analyses of factors associated with the knowledge of antimicrobial resistance (KAMR) among the farmers in Nsukka area

Parameter	Variable	Good Knowledge	Poor Knowledge	*P*
Educational Level	Secondary Education	4 (50%)	4 (50%)	.001*
	Tertiary Education	36 (100%)		
Gender	Male	32 (100 %)		.004*
	Female	8 (66.7%)	4 (33.3%)	
Farming Experience	<5years	12 (100%)		.011*
	5–10 years	10 (71.4%)	4 (28.6%)	
	>10 years	18 (100%)		
Farm Size	1(<500 birds)	12 (75%)	4 (25%)	.048*
	2(500-4999)	14 (100%)		
	3(5000-10 000)	12 (100%)		
	4(>10 000)	2 (100%)		
Type of bird Sampled	1(Layers)	26 (100%)		.001*
	2(Broilers)	10 (83.3%)	2 (16.7%)	
	3(Broiler & Cockrel)		2 (100%)	
	4(Broiler & Layer)	4 (100%)		
Type of Intensive System Practiced	1(Deep litter)	34 (89.5%)	4 (10.5%)	.627
	2(Battery cage)	6 (100%)		
Presence of other Animals	1(None)	24 (92.3%)	2 (7.7%)	1.000
	3(Goat, sheep, Pig & dog)	16 (88.9%)	2 (11.1%)	

KAMR: knowledge of antimicrobial resistance; p: probability level; %: percentage, *=statistical significance.

Table 2. Effect of socioeconomic characteristics of farmers cum knowledge of antimicrobial resistance on the frequency of antimicrobial use (FAU) among the respondents:

Parameter	Variable	Minimal Use	Extensive Use	*P*
Educational Level	Secondary Education		8 (100%)	.339
	Tertiary Education	6 (16.7%)	30 (83.3%)	
Gender	Male	4 (12.5%)	28 (87.5%)	1.000
	Female	2 (16.7%)	10 (83.3%)	
Farming Experience	<5years	4 (33.3%)	8 (66.7%)	.022*
	5 – 10 years	2 (14.3%)	12 (85.7%)	
	>10 years		18 (100%)	
Farm Size	<500 Birds	2 (12.5%)	14 (87.5%)	.184
	500 -4999 Birds	4 (28.6%)	10 (71.4%)	
	5000-10,000 Birds		12 (100%)	
	>10,000 Birds		2 (100%)	
Type of Bird Sampled	Layers	4 (15.4%)	22 (84.6%)	.903
	Broilers	2 (16.7%)	10 (83.3%)	
	Broiler and Cockrel		2 (100%)	
	Broiler and Layer		4 (100%)	
Type of Intensive System Practiced	Deep Litter System	4 (10.5%)	34 (89.5%)	.182
	Battery Cage System	2 (33.3%)	4 (66.7%)	
Presence of other Animals	None	6 (23.1%)	20 (76.9%)	.067
	Goat, Sheep, Pig and dog		18 (100%)	
Knowledge of Antimicrobial Resistance (KAMR)	Poor Knowledge		4 (100%)	.627
	Good Knowledge	6 (15%)	34 (85%)	

FAU: frequency of antimicrobial use; p: probability level; %: percentage, *= statistical significance.

Table 3. Effect of socioeconomic characteristics of farmers cum knowledge of antimicrobial resistance on the purpose of antimicrobial use (PAU) among the respondents

Variable	Characteristics	Treatment	Treatment&growth promotion	*P*
Educational Level	Secondary Education		8 (100%)	1.000
	Tertiary Education	2 (5.6%)	34 (94.4%)	
Gender	Male	2 (6.25%)	30 (93.75%)	.594
	Female		12 (100%)	
Farming Experience	<5years		12 (100%)	.328
	5 – 10 years		14 (100%)	
	>10 years	1 (11.1%)	16 (88.9%)	
Farm Size	<500 Birds		16 (100%)	.160
	500 -4999 Birds		14 (100%)	
	5000-10,000 Birds	2 (16.7%)	10 (83.3%)	
	>10,000 Birds		2 (100%)	
Type of Bird Sampled	Layers	2 (7.7%)	24 (92.3%)	.670
	Broilers		12 (100%)	
	Broiler and Cockrel		2 (100%)	
	Broiler and Layer		4 (100%)	
Type of Intensive System Practiced	Deep Litter System	2 (5.3%)	36 (94.7%)	1.000
	2 Battery Cage System		6 (100%)	
Presence of other Animals	1 None		26 (100%)	.162
	3 Goat, Sheep, Pig and dog	2 (11.1%)	16 (88.9%)	
Knowledge of Antimicrobial Resistance (KAMR)	Poor Knowledge		4 (100%)	1.000
	Good Knowledge	2 (5%)	38 (95%)	

PAU: purpose of antimicrobial use; p: probability level; %: percentage, *=statistical significance.

Table 4. Effect of socioeconomic characteristics of farmers cum knowledge of antimicrobial resistance on the disposal of dead animals (DDA) among the respondents:

Parameter	Variable	Poor Practice	Good Practice	*P*
Educational Level	Secondary Education	4 (50%)	4 (50%)	1.000
	Tertiary Education	16 (44.4%)	20 (55.6%)	
Gender	Male	14 (43.75%)	16 (56.25%)	.746
	Female	6 (50%)	6 (50%)	
Farming Experience	<5years	6 (50%)	6 (50%)	1.000
	5 – 10 years	6 (42.9%)	8 (57.1%)	
	>10 years	8 (44.4%)	10 (55.6%)	
Farm Size	<500 Birds	10 (62.5%)	6 (37.5%)	.031*
	500 -4999 Birds	6 (42.9%)	8 (57.1%)	
	5000-10,000 Birds	2 (16.7%)	10 (83.3%)	
	>10,000 Birds	2 (100%)		
Type of Bird Sampled	Layers	10 (38.5%)	16 (61.5%)	.245
	Broilers	8 (66.7%)	4 (33.3%)	
	Broiler and Cockrel		2 (100%)	
	Broiler and Layer	2 (50%)	2 (50%)	
Type of Intensive System Practiced	Deep Litter System	16 (42.1%)	22 (57.9%)	.387
	Battery Cage System	4 (66.7%)	2 (33.3%)	
Presence of other Animals	None	14(53.85%)	12 (46.15%)	.227
	Goat, Sheep, Pig and dog	6 (33.3%)	12 (66.7%)	
Knowledge of Antimicrobial Resistance (KAMR)	Poor Knowledge	2 (50%)	2 (50%)	1.000
	Good Knowledge	18 (45%)	22 (55%)	

DDA: disposal of dead animals; p: probability level; %: percentage, * = statistical significance.

Table 5. Effect of sociodemographic characteristics of farmers cum knowledge of antimicrobial resistance on the disposal of drug packs (DDP) among respondents:

Parameter	Variable	Poor Practice	Good Practice	*P*
Educational Level	Secondary Education	4 (50%)	4 (50%)	.185
	Tertiary Education	28 (77.8%)	8 (22.2%)	
Gender	Male	24 (75%)	8 (25%)	.707
	Female	8 (66.7%)	4 (33.3%)	
Farming Experience	<5years	10 (83.3%)	2 (16.7%)	.631
	5 – 10 years	10 (71.4%)	4 (28.6%)	
	>10 years	12 (66.7%)	6 (33.3%)	
Farm Size	<500 Birds	12 (75%)	4 (25%)	.085
	500 -4999 Birds	12 (85.7%)	2 (14.3%)	
	5000-10,000 Birds	8 (66.7%)	4 (33.3%)	
	>10,000 Birds		2 (100%)	
Type of Bird Sampled	Layers	18 (69.2%)	8 (30.8%)	.051*
	Broilers	10 (83.3%)	2 (16.7%)	
	Broiler and Cockrel		2 (100%)	
	Broiler and Layer	4 (100%)		
Type of Intensive System Practiced	Deep Litter System	28 (73.7%)	10 (26.3%)	1.000
	Battery Cage System	4 (66.7%)	2 (33.3%)	
Presence of other Animals	None	18 (69.2%)	8 (30.8%)	.733
	Goat, Sheep, Pig and dog	14 (77.8%)	4 (22.2%)	
Knowledge of Antimicrobial Resistance (KAMR)	Poor Knowledge		4 (100%)	.004*
	Good Knowledge	32 (80%)	8 (20%)	

DDP: disposal of drug packs; p: probability level; %: percentage; * = statistical significance.

Table 6. Adjusted logistic regression analysis of the factors associated with the farmers knowledge of antimicrobial resistance (KAMR), and practices on antimicrobial use (DDP, PAU & DDA).

Variables	Knowledge	Practices		
	KAMR	PAU	DDP	DDA
	OR, 95% CI, p	OR, 95% CI, p	OR, 95% CI, p	OR, 95% CI, p
Gender	9.113, 0.923-89.945, 0.059	0.022,0.011-2.313, 0.375	0.581, 0.032-10.707, 0.715	0.251, 0.251-26.793, 0.424
Level of Education	70.210, 4.646-161.005, 0.002*	0.922, 0.754-4.211, 0.495	1.108, 0.062-19.857, 0.945	0.920, 0.071-11.974, 0.949
Farming Exp	0.235, 0.030-1.822, 0.166	0.784, 0.032-2.910, 0.125	2.791, 0.669-11.639, 0.159	0.550, 0.147-2.052, 0.373
Layers	7.250, 0.257-204.146, 0.245	0.000, 0.000-0.000, 0.998	0.429, 0.008-23.129, 0.677	0.628, 0.026-14.900, 0.774
Broilers	0.634, 0.055-7.271, 0.715	0.431, 0.305-3.041, 0.375	0.245, 0.004-15.889, 0.508	0.240, 0.007-8.010, 0.425
Farm Size	2.220, 0.456-10.824, 0.324	0.000, 0.000-0.000, 0.998	2.640, 0.670-10.404, 0.165	1.603, 0.590-4.353, 0.355
Type of Intensive System	0.000, 0.000-0.000, 0.999	0.379, 0.217-2.447, 0.565	3.199, 0.146-69.838, 0.460	2.115, 0.161-27.799, 0.569
Other Animals Reared	2.703, 0.222-32.964, 0.436	0.000, 0.000-0.000, 0.997	0.082, 0.004-1.821, 0.114	3.207, 0.264-38.955, 0.360
KAMR		0.833, 0.023-2.110, 0.125	0.057, 0.002-1.380, 0.078	0.948, 0.055-16.476, 0.971

KAMR: knowledge of antimicrobial resistance; DDP: disposal of drug packs; PAU: purpose of antimicrobial use; DDA: disposal of dead animals; OR: odds ratio; CI: confidence interval; p: probability level; %: percentage.

DISCUSSION

The present study reveals that poultry farmers who had tertiary education demonstrated a commendable level of understanding regarding resistance to antimicrobials. This finding aligns with some previous research outcomes.[16,19] Attaining high level of education has been reported to expose

farmers to veterinary facilities, good farm management, adopting biosecurity measures and better understanding of the use of antimicrobials.[20] On the contrary, farmers with low level of education are more likely to depend on self-help rather than consult professionals, thereby increasing the chances of antibiotic misuse and the resultant AMR development.[16] Although a good number of the respondents in this study had good knowledge of AMR, it did not translate to good farm practices. Hence, though most of the farmers were aware that non-observance of the withdrawal period could result in antimicrobial resistance, yet only 18.18% practiced it. This could be ascribed to the fact that most farmers are more concerned with profit rather than the health of the consumers. Such mindset can only be corrected by enforcement of legislations on antimicrobial use as well as advocacy.

This study also found knowledge of antimicrobial resistance to be positively associated with farm size with respondents with increased farm size having better knowledge. Given that much capital is invested in setting up large farms, such farms usually employ or consult professionals (veterinarians) to ensure prudent antimicrobial use in order to protect the investment. This is unlike small-scale poultry farms where little capital is invested, and given that antimicrobial use is poorly controlled in Nigeria, most of these rely on unauthorized drug sellers for prescription and purchase of antibiotics, which is usually not devoid of abuse.[16]

The study also found good knowledge of antimicrobial resistance to be associated with gender with more of the males having good knowledge than the females. This may be attributed to the fact that majority of the males (59%) had tertiary education, and therefore were more likely to be exposed to information on antimicrobial resistance. In line with this, another study[15] also a reported male poultry farmers showing better understanding of antimicrobial resistance. Furthermore, keeping only layers was also found to be a factor in having good understanding of antimicrobial resistance. This observation may be credited to the fact that layer farmers are more likely than broiler farmers to consult with veterinarians when selecting antimicrobials.[16] In addition, layers are known to take longer time to reach the production level and as well last longer. As a result, rearing of layers necessitates greater investment, experience, and knowledge. Farmers who raise layers are therefore more likely to observe antimicrobial resistance given that the longer the birds last in the farms the more the likelihood of exposure to infections and the accompanied antimicrobial treatment and possible experiencing of antimicrobial resistance. This aligns with the fact that this study also found farming experience to be associated with the knowledge of AMR. The study found the more experienced farmers to have better knowledge of AMR than those with fewer years of experience. This finding is consistent with the report of other researchers.[16,21] Increased years of experience among poultry farmers, according to Hassan et al.[16], may result to proficiency in poultry farming, insight for exploring veterinary services, and involvement in continuous training, and awareness programs gearing towards AMU and AMR.

Ironically, having an appreciable year of experience in the poultry industry did not translate into responsible use of antimicrobials, among the respondents. All categories of farmers, including those who had spent more than ten years in the business were involved in extensive use of antimicrobial agents. This finding lends credence to the notion that some poultry farmers turn to antibiotic doping as a quick fix for poor management practices.[4] Regrettably, improper disposal of expired or used drug packs was observed even among some poultry farmers with good knowledge of antimicrobial resistance. This finding contradicts previous belief which stated that improved education and knowledge are positive predictors of behavioral changes among farmers battling AMR.[16]

The work also observed better disposal of dead birds among farmers with higher capacity farms. It is not surprising to observe proper disposal of dead animals among farmers with higher flock sizes. This could be attributed to the fact that with more to loss in the case of disease outbreak and the fact that such farms are more likely to employ the services of experts in poultry production, they seem to be more careful with the handling and disposal of dead birds.

This study found that most of the farms surveyed lacked basic hygienic and biosecurity procedures, despite most of the farmers having tertiary education and had been in production for more than a decade. Biosecurity measures in animal husbandry refer to a variety of actions taken to prevent the introduction and spread of infectious agents on the farm [22]. Basic biosecurity measures include limiting the presence of other farm animals, rodents, and insects; curtailing unlimited access to the poultry pen; enforcing strict hygienic rules such as handwashing; changing boots and overalls before entering the pen; as well as using footbaths containing disinfectants among others. Such poor farm biosecurity and hygiene practices have been linked to AMR due to increased antimicrobial use arising from the incessant exposure of the farm animals to infections.[23] Therefore, controlling antimicrobial use in livestock farms via adequate biosecurity measures remains an effective means of curtailing the emergence of antimicrobial resistant pathogens.[24] Studies on the presence of *Escherichia coli* and *Campylobacter* in poultry

have revealed a link between poor biosecurity and the occurrence or persistence of antibiotic resistance in farms[22] and in piggeries improved biosecurity measures resulted in minimal antimicrobial use.[22] In all this, there is the danger of dissemination of antimicrobial-resistant bacteria and resistance genes via varied means, such as contaminated feed, resistance genes in animal wastes as well as transmission between farms, migrating animals, and via contaminated environment.[25] These therefore underscores the need for strict hygienic and biosecurity measures.

All the farmers interviewed in this study packed and disposed the farm dung on land. The common practice of dumping animal manure into the environment has been linked to the emergence of antimicrobial-resistant bacteria.[26] Faecal enterococci from broiler chickens, for instance, are known potential carriers of conjugal transposons that confer resistance.[14] The ongoing spread of such mobile genetic elements in the microbial environment is thus a cause for concern. Faecal contamination of ready-to-eat food products such as vegetables and fruits, which are typically consumed without prior heat treatment, as well as the risk of farm and abattoir workers being exposed to these antimicrobial-resistant pathogens, is of serious public health concern, especially in developing countries. This is particularly true, given the poor level of sanitary measures adopted in farms, slaughterhouses and food processing facilities in Nigeria.[27] Given that many farms and slaughter houses are channeled into water bodies in Nigeria, these serve as sources of contamination of seafood and products, which serve as suitable substrates for microbial growth when such faeces are washed into the water bodies, and pose public health threats.[28]

Quite a good number of the farmers used antimicrobials for growth promotion, prophylactic, and therapeutic purposes. For decades, the use of antimicrobials to enhance growth has been contentious.[29] High doses and/or indiscriminate use of antimicrobials for therapeutic, preventive, and non-therapeutic purposes, culminate in the build-up of drug residues in the edible components of treated animals, and has been linked to allergic reactions, carcinogenicity, and the development of AMR.[10] Farmers, as observed in this study, were involved in non-therapeutic antimicrobial usage as an "easy fix" or compensation for poor management practices and to increase profits. As earlier stated, antibiotic doping for prophylaxis or growth acceleration is detrimental to public health and cannot replace effective farm management practices that encourage rigorous biosecurity, routine immunization, and proper nutrition.[4] To curb incessant antimicrobial use, measures such as drug licensing, drug use surveillance,[21] and compulsory testing of food of animal origin for drug residues and punishment of offenders should be implemented. Finally, poor disposal of used or expired drug packs was also practiced by the farmers who reared either layers or broilers, or both. Improper disposal of unused antimicrobials result to the accumulation of antimicrobial resistant bacteria and resistance genes in the environment recognized as an emerging pollutant.[30] Proper disposal of antimicrobials therefore remains an important aspect of the drug management cycle. There should be continuous education in the forms of town hall meetings, radio jingles and the use of social media targeting the farmers in the study area to put into their consciousness that antimicrobial resistance originates from the misuse and poor handling of drugs.

In conclusion despite good knowledge of antimicrobial resistance, a significant proportion of farmers encountered in this study engaged in poor biosecurity measures, indiscriminate use of antimicrobials for prophylaxis and growth enhancement, as well as poor adherence to withdrawal periods. Therefore, proper sanitary measures and adequate biosecurity measures in farms, especially poultry farms, are of utmost importance. Strict adherence to policies on the use of antimicrobials in livestock production should be adopted in the study area.

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Detection and Molecular Characterization of Salmonella Enterica Serovar Enteritidis in Household Chicken Eggs: A Case Study From Erzurum, Türkiye

Berna YANMAZ[1]
Ediz Kağan ÖZGEN[2]

[1]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Public Health, Burdur, Türkiye
[2]Atatürk University, Faculty of Veterinary Medicine, Department of Veterinary Microbiology, Erzurum, Türkiye

Aile Tipi Tavuk Yumurtalarında Salmonella Enterica Serovar Enteritidis'in Tespiti ve Moleküler Karakterizasyonu: Erzurum İli, Türkiye'den Bir Vaka Çalışması

ABSTRACT

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is the most predominant serovar in poultry and eggs, and it poses a significant threat to public health worldwide as it is a leading cause of salmonellosis in humans, which is transmitted through the consumption of contaminated poultry products like eggs. The objective of this study was to evaluate the presence of *S.* Enteritidis in household chicken eggs collected from Erzurum Province, Türkiye. A total of 168 household chicken eggs were collected from 168 small family poultry farms. *Salmonella spp.* was not isolated from any of the examined egg internal contents; however, successful isolation and identification of *Salmonella spp.* were achieved in 4 of the shell samples. Further characterization of the identified *Salmonella spp.* isolates was performed at the serovar level using 16S rDNA sequence analysis, and all 4 isolates were identified as *S.* Enteritidis. In conclusion, this study highlights the inherent risk of *S.* Enteritidis contamination in household chicken eggs and emphasizes the vital significance of implementing stringent food safety measures to safeguard consumer well-being and ensure the protection of public health.

Keywords: Chicken, food safety, molecular, *Salmonella* Enteritidis, 16S rDNA

ÖZ

Salmonella enterica serovar Enteritidis (*S.* Enteritidis), kümes hayvanları ve yumurtalarda en yaygın serovardır. Dünya genelinde halk sağlığı için önemli bir tehdit oluşturmaktadır çünkü insanlarda salmonellozun önde gelen nedenidir. Kontamine tavuk ürünleri, özellikle yumurtaların tüketimi yoluyla insanlara bulaşır. Bu çalışmanın amacı, Türkiye'nin Erzurum ilinden toplanan evcil tavuk yumurtalarında *S.* Enteritidis'in varlığını değerlendirmektir. Toplam 168 evcil tavuk yumurtası, 168 adet küçük aile tipi tavuk çiftliğinden toplandı. *Salmonella* spp., incelenen hiçbir yumurta içeriğinden izole edilmedi. Ancak *Salmonella spp*.'nin başarılı izolasyonu ve tanısı 4 kabuk örneğinde elde edildi. Tanımlanan *Salmonella spp*. izolatlarının serovar düzeyinde daha fazla karakterizasyonu için 16S rDNA sekans analizi kullanıldı. Tüm izolatlar *S.* Enteritidis olarak tanımlandı. Sonuç olarak, bu çalışma ev tipi tavuk yumurtalarında *S.* Enteritidis kontaminasyon riskini ortaya koymakta, tüketicinin sağlığını korumak ve halk sağlığını güvence altına almak için sıkı gıda güvenliği önlemlerinin uygulanmasının hayati önemini vurgulamaktadır.

Anahtar Kelimeler: Tavuk, gıda güvenliği, moleküler, *Salmonella* Enteritidis, 16S rDNA

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Corresponding author/Sorumlu Yazar:
Berna YANMAZ
E-mail: bernayanmaz@gmail.com

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INTRODUCTION

Salmonella enterica serovar Enteritidis (*S*. Enteritidis) is a prominent serovar commonly found in poultry and eggs, representing a significant public health concern worldwide.[1] Consumption of contaminated eggs, has been identified as a primary mode of transmission for salmonellosis in humans.[2] Traditional microbiological methods for detecting Salmonella in eggs and egg products typically require around five days.[3] However, these methods are time-consuming, labor-intensive, and expensive. They involve the isolation and cultivation of bacteria followed by biochemical and serological identification, which can be prone to false-negative results or misidentification. Additionally, the prolonged time required for analysis hinders timely intervention and control measures. On the other hand, Polymerase Chain Reaction (PCR) provides highly specific and sensitive tools for confirming the presence of *S.* Enteritidis. PCR targets specific regions of the bacterial genome and amplifies them, allowing for rapid detection and identification. This method has been widely utilized in various studies for its ability to detect *S*. Enteritidis with high accuracy.[4,5]

Genotyping methods play a pivotal role in characterizing the genetic diversity and relatedness of *S.* Enteritidis isolates.[6] These techniques enable the differentiation and classification of bacterial strains based on their genetic profiles, facilitating epidemiological investigations and providing insights into the transmission dynamics of the pathogen. One commonly used genotyping approach involves the amplification and sequencing of the 16S rDNA gene region. The 16S rDNA gene region is highly conserved among bacteria, making it an ideal target for genotyping studies.[7] The gene region contains both conserved regions that allow for primer design and variable regions that provide discriminatory power for distinguishing between different *S.* Enteritidis strains.[8]

The number of laying hens in Türkiye has reached a staggering 124 million, positioning the country as the 8th largest producer of hen eggs worldwide, with a production volume of 1.2 million tons.[9] The registration of backyard poultry flocks in Türkiye is currently a voluntary practice undertaken by various hobby and ornamental poultry associations. As a result, there is a lack of precise information regarding the number of owners, flock sizes, locations, and management practices. Moreover, with the growing prevalence of backyard poultry farming in urban areas, regional authorities often face difficulties in establishing a comprehensive legal framework to address the situation effectively. Municipalities throughout the country are encountering challenges in regulating backyard poultry farming within residential areas due to the absence of enforceable legislation in Türkiye that delineates the guidelines for raising poultry in such settings.[10] Erzurum Province, located in Türkiye, is renowned for its widespread adoption of house-type poultry farming, which highlights the importance of conducting comprehensive surveillance and characterization of *S.* Enteritidis in this area. The objective of this study was to evaluate the presence of *S.* Enteritidis in household chicken eggs collected from Erzurum Province, Türkiye. Moreover, PCR and genotyping techniques were employed for the confirmation, identification, and categorization of isolates by conducting 16S-rDNA gene sequencing.

MATERIALS AND METHODS

Animals and Experimental Design

The Atatürk University Local Ethical Committee approved the study with a protocol (Date: 19/10/2022). A total of 168 household chicken eggs were collected from 168 small family poultry farms in Erzurum province, Türkiye, between January and March 2023 (Figure 1). These farms primarily produce eggs for their own consumption. The characteristics of the poultry farms included having less than 40 animals, with the majority of chicks hatching through the natural brooding process. The animals were allowed to roam freely and interact with neighboring habitations, and no prophylaxis or veterinarian controls were implemented for the animals. The sampling achieved directly from the depository. The eggs were collected in sterile containers and transported to the laboratory under refrigerated conditions and processed on the same day.

Figure 1. The geographic location of Erzurum province, Türkiye.

Isolation and Detection of Salmonella

The egg samples collected were subjected to separate culture processes for both the eggshell and egg content, following the method described earlier.[11] To initiate the process, sterile swabs were moistened by dipping them in sterile peptone water and then applied to the entire eggshell. Subsequently, the swab samples were transferred into 4 mL of peptone water. Next, the eggs underwent sterilization by being fully immersed in 2% tincture iodine for 1 minute. Following sterilization, the eggs were transferred into sterile bags, where they were broken and

mixed with 50 mL of peptone water. The egg yolk was thoroughly mixed until it achieved complete dispersion and homogeneity. Subsequently, a 1 mL sample was extracted from the mixture and transferred into 4 mL of peptone water.

The samples of eggshell and egg content were incubated at a temperature of 37°C for a duration of 24 hours. Following the incubation period, 100 µL of each peptone water sample was extracted and transferred into Rappaport Vassiliadis broth, which was then incubated at a temperature of 42°C for another 24 hours. Subsequently, loopfuls of both cultures were streaked onto selective agar plates, including Brilliant Green agar (BG), Xylose Lysine Deoxycholate agar (XLD), and Salmonella-Shigella agar (SS). The agar plates were then incubated at 37°C for 24 hours. Presumptive colonies were selected from each plate and subsequently subcultured in TSI slant medium and various differential culture media, including MR-VP broth, urea agar, SIM agar, and Simmon's citrate agar. The subcultures were then incubated at 37°C for 24 hours. A single colony of Salmonella spp. isolates was suspended in 500 µL of phosphate-buffered saline. Meanwhile, a colony was placed in TSB supplemented with 15% glycerin and stored at -20°C for subsequent analysis.

DNA Extraction

A commercial ready-made nucleic acid isolation kit (Qiagen, Hilden, Germany) was utilized for DNA extraction. The DNA extraction process was carried out on Salmonella spp. isolates suspended in PBS, which were in the culture stage. The resulting nucleic acids were subsequently stored at -20°C for analysis. Molecular identification and phylogenetic analysis of the isolated Salmonella spp. isolates were performed using 16S rDNA analysis.

PCR Amplification and DNA Sequence Analysis

Salmonella spp. isolated in culture were subjected to DNA sequence analysis. To distinguish each Salmonella spp. isolate based on the order of isolation, a unique "chicken egg" (CE) code was assigned to each isolate. Prior to sequence analysis, genomic DNA extraction was performed using the cador pathogen mini kit (Qiagen, Hilden, Germany). The quantity and purity of the extracted nucleic acids were assessed using Thermo Scientific Nanodrop 2000. Subsequently, the 16S rDNA gene region was amplified for DNA sequencing using 27F and 1492R primers, as described earlier.[12] PCR reactions were conducted in 0.2 ml tubes with a final volume of 35 µL. Each reaction contained 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.3 µM of each primer, 2 U of Taq DNA polymerase, and 3 µL of template DNA. The remaining volume was made up to 35 µL using sterile distilled water. The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and elongation at 72°C for 1 minute. A final elongation step was performed at 72°C for 5 minutes. The DNA sequence analysis was carried out on the obtained amplicons to determine the nucleotide sequence information.

DNA sequencing was performed using the Oxford Nanopore Technologies® MinION™ Mk1C device. The Rapid Barcoding Kit 96 (SQK-RBK110.96) was utilized for sequence analysis. DNA samples were measured using the Qubit 1X dsDNA BR (Broad Range) Assay Kit. Each DNA sample was extracted and transferred to a sterile microcentrifuge tube with a volume of 1.5 ml, and the volume was adjusted to 9 µl with sterile distilled water. Barcodes were added to the prepared DNA samples separately, with 1 µl for each. For barcoding, heat treatment was applied at 30°C for 2 minutes and at 80°C for 2 minutes. Following the heat treatment, all barcoded DNAs were combined in the same microcentrifuge tube, and library preparation was conducted according to the kit protocol.

After library preparation, loading was performed onto the MinION Flow Cell (R9.4.1), which was attached to the Oxford Nanopore Technologies® MinION™ Mk1C device, and sequencing was conducted. The sequencing process involved obtaining a minimum of 300 reads for each barcode, and the resulting Fastq and Fast5 files were analyzed using Geneious Prime 2022.1.1. From the analysis, the consensus sequence data obtained was subjected to NCBI BLAST analysis. The genetic relationships between the obtained 16S rRNA genes and representative strains of Salmonella spp. were inferred using the MEGA X software, employing the maximum likelihood method based on Kimura 2-parameter model with 1000 bootstrap.

RESULTS

The analysis revealed that *Salmonella spp*. was not isolated from the internal contents of the egg samples. However, *Salmonella spp*. was found to be isolated from only 2.3% (4/168) of the outer surface samples of the examined eggs (Figure 2).

The CE1 DNA sample showed 100% similarity with *S*. Enteritidis strain SEO, identified by the Sequence ID CP033090.1 in the NCBI Blast search. The CE2 DNA sample exhibited 99.93% similarity with *S*. Enteritidis strain NCM 61 (Sequence ID: CP032851.1), *S*. Enteritidis strain SEE2 (Sequence ID: CP011791.1), and *S*. Enteritidis strain SEE1 (Sequence ID: CP011790.1). The analysis of the CE3 labeled strain's 16S rDNA identified it as an isolate that shared the

highest similarity (100%) with a bacterial sequence. *S*. Enteritidis strain MFDS1018147 exhibited 100% similarity to the CP110220.1 gene region, *S*. Enteritidis strain MASJG9 to the OP744581.1 gene region, *S*. Enteritidis strain R17.1476 to the CP100724.1 gene region, *S*. Enteritidis strain R18.1630 to the CP100666.1 gene region, *S*. Enteritidis strain SE006 to the CP099973.1 gene region, *S*. Enteritidis strain PNUSAS034908 to the CP092321.1 gene region, *S*. Enteritidis strain CVM N17S192 to the CP082726.1 gene region, *S*. Enteritidis strain CVM N17S111 to the CP082729.1 gene region, *S*. Enteritidis strain CFSAN051827 to the CP075122.1 gene region, *S*. Enteritidis strain CFSAN051882 to the CP075120.1 gene region, *S*. Enteritidis strain CFSAN022640 to the CP075019.1 gene region, *S*. Enteritidis strain CFSAN008104 to the CP074661.1 gene region, *S*. Enteritidis strain CFSAN051890 to the CP075118.1 gene region, *S*. Enteritidis strain CFSAN026631 to the CP074254.1 gene region, and *S*. Enteritidis strain CFSAN026633 to the CP074252.1 gene region, all with 100% similarity. The analysis of the CE4 isolate revealed a 100% similarity to the CP007245.1 gene region of *S*. Enteritidis strain EC20120008.

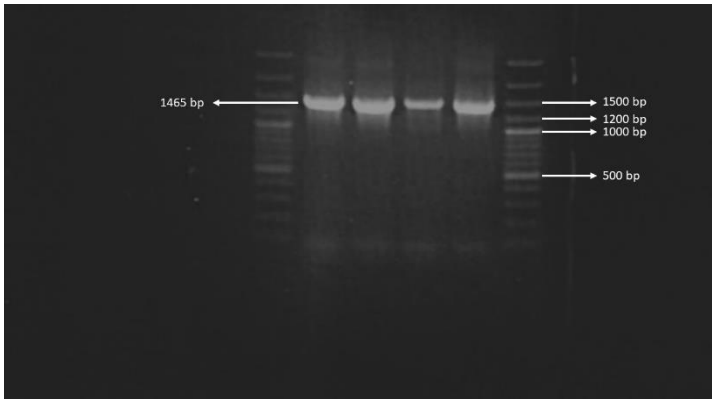

Figure 2. The image of positive S. Enteriditis PCR reactions is shown. In the agarose gel image of S. Enteriditis positive egg samples; the reference indicator (1st place marker (100-3000 bp), second (CE1), third (CE2), fourth (CE3), and fifth (CE4) are 1465bp bands belonging to positive samples.

The neighbor-joining phylogenetic tree generated using Mega X software is shown in Figure 3. The DNA sequences determined in the analysis have been uploaded to the NCBI GeneBank, and accession numbers were obtained for the CE1, CE2, CE3, and CE4 isolates, respectively, as OQ108763.1, OQ108764.2, OQ108765.1, and OQ108766.1.

DISCUSSION

The objective of the present study was to isolate *Salmonella spp*. from both eggshells and eggs, and to perform phylogenetic characterization through 16S rDNA sequence analysis, utilizing egg samples collected from the Erzurum province.

Figure 3. Phylogenetic tree based on the nucleotide sequences of 16S rDNA genes. The tree was constructed by the neighbor-joining method, using the computer program (Mega X software).

The prevalence of *Salmonella spp.* in eggs across European Union countries is reported to be 0.8%,[13] while in France, the prevalence of *Salmonella spp*. on eggshells was found to be 1.05%.[14] In China, 7.1% of *Salmonella spp*. was isolated from eggshells intended for consumption, and 8.3% from the internal contents of the eggs.[15] Notably, a study conducted in Iran reported a detection rate of 1.33% for *Salmonella spp*. on eggshells, whereas *Salmonella spp*. could not be isolated from the egg content.[16] In a study carried out in Poland, *Salmonella spp*. could not be isolated from both the shell samples and the egg content of 1200 eggs.[17] Conversely, in India, *Salmonella spp.* was detected on 6.1% of eggshells and in 1.8% of the egg content.[18] In the presented study, the observed isolation rate from both eggshells and egg content is consistent with findings from similar studies.[13,14]

The contamination of eggs with *Salmonella spp.* can occur through two distinct routes: horizontal transmission, which involves colonization of the digestive tract and subsequent contamination with feces, or vertical transmission, wherein the reproductive organs become infected with *Salmonella spp*.[13] The study's specific isolation from eggshells suggests the occurrence of horizontal transmission.

More than 90% of *Salmonella* species isolated in European countries are reported to be *S.* Enteritidis.[13] Similarly, a study conducted in India found that 86% of the serotypes isolated from eggshells were *S*. Enteritidis.[18] The results of our study conducted in the province of Erzurum indicate that the isolation of only *S.* Enteritidis from eggshells is consistent with the literature.

The findings from our analysis of the DNA samples obtained from different *S.* Enteritidis strains provide valuable insights into the genetic characteristics and relatedness of these isolates. The CE1 and CE2 isolates exhibited significant degrees of similarity, indicating a potential genetic lineage or shared ancestry with the reference strains. These reference strains include SEO (unpublished data), isolated from human feces in China, NCM 61 isolated from chicken meat in China, and SEE2 and SEE1 (unpublished data), both isolated from eggshells. Close genetic similarity was observed between the CE3 isolate from traditional Japanese food (CP110220.1=unpublished) in South Korea, broiler chicken secal content in Pakistan (OP744581=unpublished), human feces (CP100724=unpublished) in Taiwan, chicken (CP099973) in Taiwan,[19] human feces in the USA,[20] chicken meat in the USA,[21] and mouse, goose, duck, domestic pig, and spinach samples in the USA (CP075122, CP075019). Furthermore, these isolates exhibited a close genetic similarity to *S.* Enteritidis isolates CP074254, CP075118, CP074252, CP074661, and CP075120 (unpublished). The remarkable similarity of the CE3 isolate with strains isolated from diverse sources across different geographic locations highlights its significance for public health. Furthermore, the 100% similarity observed between the CE4 isolate and the gene region of *S*. Enteritidis strain EC20120008, isolated from a reptile in Canada by previous report,[22] suggests a potential clonal relationship between these two isolates. These findings enhance our understanding of the genetic characteristics of *S.* Enteritidis strains and provide a foundation for future studies on their epidemiological and pathogenic properties.

Despite the valuable insights provided by our analysis into the *S.* Enteritidis strains, it is important to acknowledge certain limitations that should be considered when interpreting the results. Firstly, the identification of *S.* Enteritidis strains based on genetic similarity through the NCBI Blast search and gene region analysis has its inherent limitations. The method relies on the available reference sequences in the database, and the accuracy of the identification is dependent on the comprehensiveness and quality of the reference database. Incomplete or insufficient representation of *S.* Enteritidis strains may lead to potential misidentification or incomplete characterization. Secondly, the analysis focused on a limited number of samples (CE1, CE2, CE3, and CE4 isolates). While these samples provided valuable information, they may not be fully representative of the overall *S*. Enteritidis population in the region. The genetic diversity and prevalence of different strains may vary, and additional samples from different sources and regions would provide a more comprehensive understanding of the *S*. Enteritidis population dynamics. Furthermore, the study employed specific molecular techniques, such as NCBI Blast search and gene region analysis, to assess genetic similarity and identify strains. While these techniques are widely used and reliable, they have their own limitations. Other genetic analysis methods, such as whole-genome sequencing, could provide more detailed information about the genetic characteristics, virulence factors, and potential antimicrobial resistance profiles of the strains. Future studies incorporating these advanced techniques would enhance the understanding of *S.* Enteritidis diversity and its implications. Lastly, the phylogenetic tree generated using the Mega X software represents a visual representation of the genetic relationships among the analyzed strains. However, the accuracy and reliability of the tree are dependent on the quality of the input data and the chosen methodology. Alternative phylogenetic analysis methods or additional statistical support would strengthen the robustness of the findings.

In conclusion, our analysis demonstrated significant genetic similarities between the tested samples and various strains of *S.* Enteritidis. These findings suggest the presence of closely related strains in the studied region. However, it is important to note that genetic similarity does not necessarily imply identical characteristics in terms of virulence or antimicrobial resistance. Future studies should focus on further characterizing these strains to gain a more comprehensive understanding of their phenotypic traits and their potential impact on public health.

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Elucidating the Molecular Mechanism by Which Gallic Acid Alleviates Oxidative Stress and Inflammatory Response of Acrylamide-Induced Renal Injury in a Rat Model

Bir Rat Modelinde Gallik Asidin Oksidatif Stresi ve Akrilamid Kaynaklı Böbrek Hasarının İnflamatuar Yanıtını Hafiflettiği Moleküler Mekanizmanın Aydınlatılması

Samet TEKİN[1]
Yusuf DAĞ[1]
Merve BOLAT[1]
Emin ŞENGÜL[1]
Mohamad WARDA[1]

[1]Atatürk University, Faculty of Veterinary Medicine, Department of Physiology, Erzurum, Türkiye

ABSTRACT

This study investigates the molecular effects of Acrylamide (ACR)-induced kidney damage and the potential protective role of Gallic acid (GA). Forty male rats were divided into five groups: Control, ACR, ACR+GA50, ACR+GA100, and GA100. The ACR groups received a daily oral dose of 50 mg/kg, while GA groups received 50 or 100 mg/kg oral doses for 14 consecutive days. On the 15th day, the animals were euthanized, and kidney samples were collected. The MDA, GSH, SOD, GPx, and CAT oxidative stress parameters were measured. The renal inflammatory response was evaluated by measuring the level of TNF-α, IL-1β, IL-6, NF-κB, COX-2, and IL-10. The downstream pro-apoptotic signaling pathway was resolved by measuring the levels of p38 MAPK and p53. The ACR induced renal oxidative stress with aggravated lipid peroxidation as revealed by the reduction in the levels GSH, SOD, GPx, and CAT of antioxidants while over-increase in the level of MDA, respectively. The levels of IL-1β, IL-6, NF-kB, COX-2 pro-inflammatory mediators as well as the p38 MAPK and p53 pro-apoptotic intermediates were further elevated. This increase in inflammatory response was met with marked decrease in anti-inflammatory IL-10 level. However, GA treatments- in dose dependent manner- had been demonstrated to effectively mitigate oxidative stress and reduce inflammatory responses, while also enhancing the cellular anti-inflammatory defense mechanisms. The GA can be considered as a novel protective antioxidant, anti-apoptotic drug against ACR-induced nephrotoxic insult. Further study should be performed to estimate the exact effective dose.

Keywords: Acrylamide, apoptosis, gallic acid, inflammation, nephrotoxicity

ÖZ

Bu çalışma, Akrilamid (ACR) kaynaklı böbrek hasarının moleküler etkilerini ve Galik asidin (GA) potansiyel koruyucu rolünü araştırmaktadır. Kırk erkek sıçan, Kontrol, ACR, ACR+GA50, ACR+GA100 ve GA100 olmak üzere beş gruba ayrılmıştır. ACR grupları günlük oral 50 mg/kg dozda alırken, GA grupları ise 14 ardışık gün boyunca 50 veya 100 mg/kg oral dozda almıştır. 15. gününde hayvanlar uyuşturularak böbrek örnekleri alınmıştır. MDA, GSH, SOD, GPx ve CAT oksidatif stres parametreleri ölçülmüştür. Böbrek iltihabi yanıtı, TNF-α, IL-1β, IL-6, NF-κB, COX-2 ve IL-10 seviyeleri ölçülerek değerlendirilmiştir. Aşağı akım pro-apoptotik sinyal yolakları, p38 MAPK ve p53 seviyeleri ölçülerek çözümlenmiştir. ACR, antioksidanların GSH, SOD, GPx ve CAT seviyelerinde azalma ve MDA seviyesinde artış ile belirginleşen lipid peroksidasyonunu şiddetlendirerek renal oksidatif stresi tetiklemiştir. IL-1β, IL-6, NF-kB, COX-2 pro-iltihabi mediatörler ve ayrıca p38 MAPK ve p53 pro-apoptotik ara maddelerin seviyeleri artmıştır. Bu artan iltihabi yanıt, anti-iltihabi IL-10 seviyesinde belirgin bir azalma ile karşılanmıştır. Bununla birlikte, doza bağlı olarak GA tedavilerinin oksidatif stresi etkin bir şekilde azalttığı, iltihabi yanıtları düşürdüğü ve hücresel anti-iltihabi savunma mekanizmalarını artırdığı gösterilmiştir. GA, ACR kaynaklı böbrek hasarına karşı yeni bir koruyucu antioksidan ve anti-apoptotik ilaç olarak değerlendirilebilir. Kesin etkili dozu belirlemek için ileri çalışmalar yapılmalıdır.

Anahtar Kelimeler: Akrilamid, apoptoz, galik asit, iltihap, nefrotoksisite

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Corresponding author/Sorumlu Yazar:
Samet TEKİN
E-mail: samet.tekin@atauni.edu.tr

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INTRODUCTION

Acrylamide (ACR) is a compound containing highly reactive α-β unsaturated carbonyl groups that widely used in the synthesis of many occupational and dietary industrial polymerized products including polyacrylamides. Moreover, its frequent daily use (as water management and treatment and/or gel chromatography in molecular laboratories[1]) increases its risk of undesirable toxic consequences to humans.[2-4] Furthermore, ACR can be instantly produced during high temperature processing of carbohydrate-rich foods.[5,6] Many studies have been conducted on ACR so far. They cause damage to many tissues and organs, such as the liver[7], kidney[8], brain[9], heart[10], and testis[11].

Because of its wide range of solubility, as in water and other solvents, it fairly reaches the liver and kidney after ingestion[12-14] with marked damage to cellular genome after the binding of its metabolite Glicamide to cellular DNA.[15,16] It triggers cellular oxidative stress, while increasing ROS production with parallel drain of cellular antioxidants. This scenario consequently disrupts the antioxidant defense mechanisms involving enzymes such as SOD, GPx and CAT as well as GSH, leading to accelerated lipid peroxidation as indicated by an increase in MDA levels. Oxidative stress is closely linked to inflammation. When cells are exposed to ACR, pro-inflammatory markers increase, while anti-inflammatory markers decrease. This dual impact of oxidative stress and inflammation induced by ACR exposure in cells can trigger apoptosis.[17,18]

GA is a plant-derived polyphenol compound abundantly found in many fruits and beverages.[19] GA protects against oxidative damage and inflammation on cellular and tissue levels.[20-23] Besides it has a powerful antioxidant with free radicals scavenger properties[24], it also demonstrated antibacterial, anti-viral, and anti-cancer effects.[25]

Our study investigated its potential anti-oxidant, anti-inflammatory and anti-apoptotic roles against ACR-induced kidney damage.

MATERIALS AND METHODS

Reagents and Chemicals

Acrylamide (≥ 99%) (Cas No: 10236-47-2) and Gallic acid (Cas No: 149-91-7) were purchased by Sigma Chemical Co (St. Louis, MO). Commercially available rat ELISA kits were used to determine the level of Malondialdehyde (MDA) (Cat. No:201-11-0157) and Superoxide Dismutase (SOD) (Cat. No:201-11-0169) in renal tissue and to determine Glutathione Peroxidase (GPx) (Cat. No: 201-11-5104), Catalase (CAT) (Cat. No: 201-11-5106) and Glutathione (GSH) activities (Cat. No: 201-11-7122; SunRedBio, Shanghai-China). Interleukin-6 (IL-6) (Cat. No: 201-11-0136), Nuclear factor kappa-B (NF-κB) (Cat. No: 201-11-0288), Tumor necrosis factor-α (TNF-α) (Cat. No: 201-11-0765), cyclooxygenase-2 (COX-2) (Cat. No: 201-11-0297), interleukin-1β (IL-1β) (Cat. No: 201-11-0288), and interleukin-10 levels (IL-10) (Cat. No:201-11-0109 were analyzed in the renal tissue of rats using Commercial ELISA kits (SunRedBio, Shanghai, China). Analysis of p38 mitogen-activated protein kinases (P-38MAPK) (Cat.No: 201-11-5464) and tumor suppressor protein p53 (TP53) (Cat. No:201-11-0072) as pro-apoptotic parameters in the kidney tissue was performed with the help of commercially available ELISA kits (SunRedBio, Shanghai, China).

Experimental Animals

Sprague Dawley male rats were obtained from Atatürk University Experimental Research and Application Center. A total number of 40 rats with an average weight of 200-250 g were used. The rats were housed in ventilated rooms with ambient temperature of 25oC and humidity of 60-65% using 12-hour light/dark cycle. Animals had water and feed ad libitum during the whole experimental period. The study protocol was approved by Atatürk University Animal Experiments Local Ethics Committee (Decision No: 2021/166).

Experimental Design

The rats were divided into five equal groups. Experimental groups were formed as follows.

Control: 1 ml of saline was given intragastric (ig) for 14 days.

ACR: ACR was given at a dose of 50 mg/kg ig for 14 days.

ACR+GA50: ACR at a dose of 50 mg/kg and GA at a dose of 50 mg/kg were given ig for 14 days.

ACR+GA100: ACR at a dose of 50 mg/kg and GA at 100 mg/kg were given ig for 14 days.

GA100: GA was given at 100 mg/kg ig for 14 days.

On the 15th day, all rats were weighed and euthanized under sevoflurane anesthesia. The kidneys of all rats were washed with cold phosphate buffer and then placed in a -20 freezer until analysis.

Homogenization of Kidney Tissue

Kidney tissue samples were cut into equal small pieces and homogenized in ice-cold phosphate buffer saline adjusted to pH 7.4 in MagNA Lyser device. Then the homogenate was centrifuged at 4000 rpm at 4 ºC for 10 minutes and the supernatant was recovered and stored at – 40 ºC for further analysis.

Analysis of Oxidant and Antioxidant Enzymes

MDA and GSH levels and SOD, GPx, and CAT activities were measured in the supernatant using commercial ELISA kits.

Analysis of Inflammation Markers in Kidney Tissue

The IL-1β, IL-6, NF-κB, COX-2, IL-10, and TNF-α levels were determined after the protocol of commercial ELISA kits in the supernatant of homogenized kidney samples.

Analysis of Apoptosis Markers in Kidney Tissue

Similarly, the apoptotic biomarkers p38 MAPK and p53 levels were determined using their available ELISA kits following their manufacturer's instructions.

Statistical Analyzes

The data were analyzed using one-way ANOVA with more than two independent groups in the SPSS 20.00 statistical programming software. The obtained values were evaluated by the Tukey test. The values were expressed as mean ± standard error (± SEM), and $P < .05$ and $P < .01$ were considered significant.

RESULTS

Oxidative Stress in ACR-induced Kidney Damage

The oxidative stress parameters in ACR-induced kidney damage and the effects of GA on these parameters are shown in Figure 1 and Figure 2.

The level of MDA in the kidney of the ACR group recorded a significant rise compared with the control ($P < .01$). While the ACR+GA(50) group demonstrated a slight non-significant decrease in MDA level ($P > .05$), the ACR+GA(100) and GA(100) groups, however, showed a normalization of the lipid peroxidation level with non-significant MDA value compared with control ($P > .05$, Figure 1).

The activity of antioxidant SOD and the reserve of GSH level were both significantly ($P < .01$) decreased in the ACR group compared to the control. Despite observed ($P < .05$) fluctuation of SOD and GSH levels in ACR+GA(50) group than that of control groups, their measured levels in the ACR+GA(100) and GA(100) groups were not differed than that of control ($p>0.05$, Figure 1).

Both GPx and CAT levels showed marked decrease ($p<0.01$) in the ACR group than their level in control. There were slight increase in their levels in the ACR+GA(50), but significantly not differed than the ACR group. The GPx and CAT levels, however, recorded a non-significance difference in the ACR+GA(100) and GA(100) groups when compared with control ($P > .05$, Figure 2).

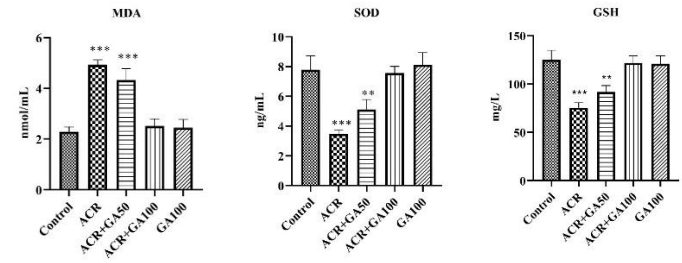

Figure 1. The effects of ACR and GA administration on MDA (A), SOD (B), and GSH (C) levels in the experimental groups (There are statistically significant differences between the values expressed with different symbols between the control group. *** $P < .01$, ** $P < .05$; n =8)

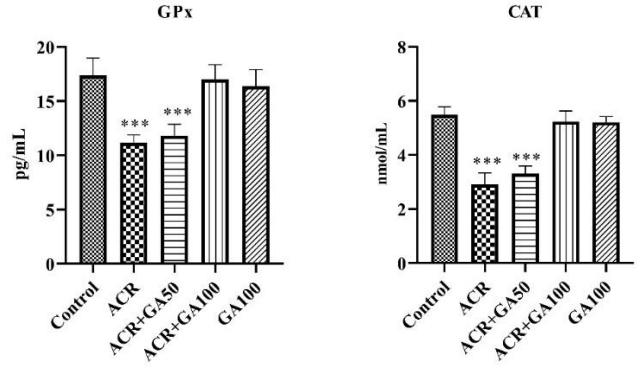

Figure 2. The effects of ACR and GA administration on GPx ve CAT levels in the experimental groups (There are statistically significant differences between the values expressed with different symbols between the control group. *** $P < .01$, n =8)

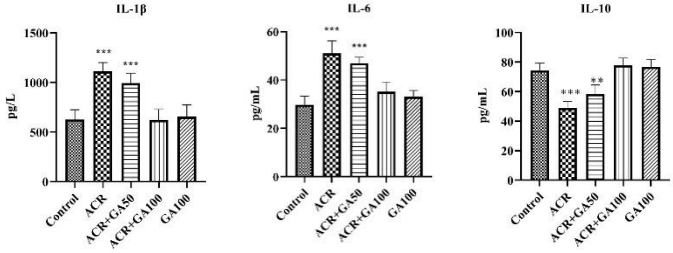

Figure 3. The effects of ACR and GA administration on IL-1β, IL-6, and IL-10 levels in the experimental groups (There are statistically significant differences between the values expressed with different symbols between the control group. ***$P < .01$, **$P < .05$, n =8)

Inflammation in ACR-induced Kidney Injury

The Some parameters related to inflammation due to ACR-induced renal insult as well as the protective role after GA treatments are shown in Figure 3 and Figure 4.

The ACR-treatment induced significant rise ($P < .01$) in IL-1β and IL-6 levels compared with control. These levels showed non-significant decrease ($P > .05$) in the ACR+GA(50) group than their level in the ACR group. The IL-1β and IL-6 levels of the ACR+GA(100) and GA(100) groups were not statistically differed than control ($P < .05$, Figure 3).

The IL-10 levels, however, not differed significantly in the ACR+GA(100) and GA(100) groups when compared with the control ($P > .01$, Figure 3). While the IL-10 level in the ACR group recorded a significant decrease ($P > .01$) in its level in comparison with the control, the ACR+GA(50) group showed a significant increase ($P < .05$) in its level than the ACR groups.

The levels of pro-apoptotic mediators NF-κB and COX-2 were significantly elevated ($P < .01$) in ACR treated group compared to the control. This elevation was not recorded in the ACR+GA(50) group, where the levels slightly decreased to be not significantly differed than that of the control level ($P < .01$). Also their levels in the ACR+GA(100) and GA(100) groups showed no statistical difference to that of control ($P > .05$, Figure 4).

Similarly, the inflammatory mediator TNF-α levels in the ACR+GA(100) and GA(100) groups were not differed significantly than the control level ($P > .05$, Figure 4). But the TNF-α level in the ACR group recorded a significant difference to the control value ($P > .01$). In the ACR+GA(50) group, however, a slight- but significant- decrease in TNF-α level that reported a significant difference ($P < .05$) to the TNF-α level of ACR group.

Apoptosis in ACR-induced Kidney Injury

The changes in the apoptotic markers were reported in Figure 5. The levels of apoptotic markers p38-MAPK and p53 increased significantly ($P < .01$) in ACR treated group compared to control. Unlike the significant change ($P < .05$) that was observed in the level of p38-MAPK between the ACR+GA (50) group and the ACR group, there was no statistical difference ($P > .05$) in the p53 level between the ACR+GA(50) group and the ACR group. The p38-MAPK and p53 levels of the ACR+GA(100) and GA(100) groups were close to the control level with no statistical difference between them ($P < .05$, Figure 5).

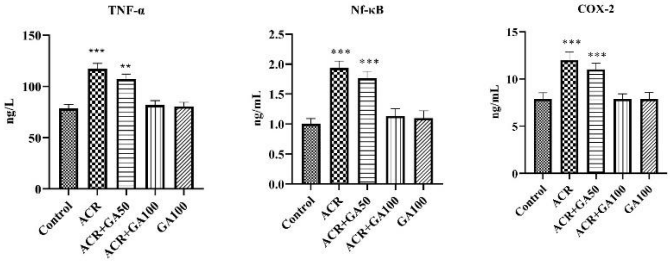

Figure 4. The effects of ACR and GA administration on TNF-α, NF-κB, and COX-2 levels in the experimental groups (There are statistically significant differences between the values expressed with different symbols between the control group. ***$P < .,01$, ** $P < .05$, n =8)

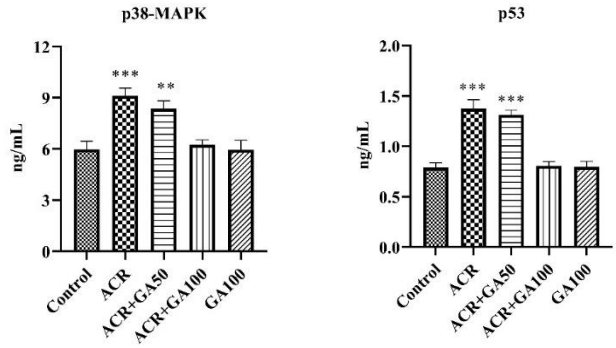

Figure 5. The effects of ACR and GA administration on TNF-α, NF-κB, and COX-2 levels in the experimental groups (There are statistically significant differences between the values expressed with different symbols between the control group. ***$P < .01$, **$P < .05$, n =8)

DISCUSSION

The study tested the potential protective role of GA in a dose dependent manner against ACR renal insult. Here the levels of oxidative stress parameters (SOD, GSH, GPx, and CAT), lipid peroxidation (MDA), inflammatory response (IL-1β, IL-6, IL-10, TNF-α ve NF-κB), and pro-apoptotic (p38-MAPK and p53) markers were determined to evaluate the possible role of GA to alleviate the oxidative stress, inflammatory response and accelerated apoptosis in the kidney tissue following ACR insult. The results confirmed that the GA- in high dose- had demonstrated a considerable mitigation of the ACR-induced renal oxidative and inflammatory insults.

Imbalances in the typical cellular redox state result in disruptions to biological components like lipids, proteins, and DNA. The extent of ROS generation determines the extent of cell membrane damage, leading to the creation of lipid peroxidation through the oxidative modification of polyunsaturated fatty acids within the membrane's composition.[10] MDA, one of the lipid peroxidation indicators, increased in our study, and GA application significantly decreased the MDA level. Oxidative stress can be defined as an imbalance between the oxidant and antioxidant defense systems.

The MDA is a convenient lipid peroxidation biomarker. Its observed elevation after ACR renal insult was significantly mitigated with oral dosing of GA. Since oxidative stress can be characterized as a disturbance in the equilibrium between the mechanisms that generate oxidants and those that provide antioxidant protection. The generation of reactive oxygen species (ROS) efficiently counteracts both enzymatic (such as SOD, GSH-Px, and CAT) and non-enzymatic (like GSH) antioxidant defenses.[25-30] While ACR

increases MDA levels, it significantly decreases SOD, CAT, GPx, and GSH levels.[31] Ghaznavi et al.[32] investigated the antioxidant role of GA in gentamicin-induced nephrotoxicity as well as the same role in methotrexate-induced hepatotoxicity.[33] Gallic had been demonstrated to modulate cellular redox hemostasis.[34] Consistently, our study proved the gallic acid role in this modulation after ACR-induced renal insult.

TNF-α, IL-1β, and IL-6 are proinflammatory cytokines, and they cause acute inflammation by stimulating the expression of adhesion molecules in endothelial cells and inflammatory cells.[35] It has been shown in a study that ACR application increases TNF-α and IL-6 levels.[36] In another study, it causes an increase in TNF-α and IL-1β levels in acrylamide-induced brain damage.[37] IL-10 is an anti-inflammatory cytokine. Acrylamide administration causes a decrease in IL-10 levels.[38] NF-κB is one of the critical transcription factors activated by oxidative stress and plays a role in inflammation. NF-κB increases the levels of IL-1β, TNF-α, and IL-6, which are involved in inflammation, and accordingly, it increases the inflammatory response. Many studies have shown that NF-κB stimulates inflammation.[39,40] NF-κB is a transcription factor involved in signal transduction between the cytoplasm and the nucleus in various cell types. COX-2 acts as an enzyme that catalyzes the oxidation of arachidonic acid. NF-κB regulates the expression of the COX-2 enzyme.[41] ACR increases COX-2 expression.[10] Gallic acid is a phenolic compound that regulates inflammation in various tissues.[6] In our study, we found that gallic acid, which has protective effects against the inflammation caused by ACR, has also a protective role against ACR-induced renal insult.

Against the stimuli outside the cell, signal pathways such as MAPK are activated inside the cell. MAPK consists of 4 subgroups. These are Extracellular Signal-Regulated Kinases (ERKs), c-Jun N-Terminal and Stress-Activated Protein Kinases (JNK/SAPK), ERK/ Big MAPK 1 (BMK1), and p38 protein kinase. To date, it is well known that p38 plays a role in apoptosis. Caspases are the best markers of apoptosis. However, caspase inhibitors inhibit p38, which means that p38 has a role in caspase activation.[42] Expression of the p53 gene is increased in DNA damage and apoptosis.[43,44] Li et al. observed that p38-MAPK and p53 levels increased in neural damage induced by hydrogen peroxide.[45] In another study, Khan et al. showed increased p38-MAPK and p53 levels in cisplatin-induced colon toxicity.[8] Gallic acid has a suppressive role on free radicals that trigger the apoptosis pathway and enzymes involved in their production.[25] Our study found that the kidney damage induced by Acrylamide increased p38-MAPK and p53, and GA had protective effects against their increase.

In conclusion, the study revealed that ACR induced renal toxicity in rats as revealed by increased oxidative stress parameters with aggravated lipid peroxidation confirmed by the rise in the levels of MDA biomarker as well as inflammatory mediators IL-1β, IL-6, TNF-α, and NF-κB. These finding were concomitant with marked drain of the cellular antioxidant activity reflected by the marked decrease in SOD, GSH, GPx, and CAT levels with a rise in the levels of pro-apoptotic markers p38-MAPK and p53. Interestingly, the use of GA that well known phenolic compound with potent antioxidant, anti-inflammatory, and anti-apoptotic properties, had been demonstrated to mitigate the adverse nephrotoxic effects induced by ACR treatment. This mitigation, however, was in a dose dependent manner. The study warrants about the possible use GA as novel pharmaceutical intervention to alleviate renal toxicity induced by ACR application.

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Investigation of the Efficacy of Sericin in Experimental Knee Osteoarthritis Model in Rats through the TGF-Beta/Smad Pathway

Deneysel Diz Osteoartrit Modeli Geliştirilen Sıçanlarda Serisinin Etkinliğinin TGF-Beta/Smad Yolağı Üzerinden İncelenmesi

Köksal GÜNDOĞDU[1]
Özgen KILIÇ ERKEK[2]
Gülşah GÜNDOĞDU[2]
Dilek SAYIN[2]
Gülçin ABBAN METE[3]

[1]Denizli State Hospital, Department of Orthopedics and Traumatology, Denizli, Türkiye
[2]Pamukkale University, Faculty of Medicine, Department of Physiology, Denizli, Türkiye
[3]Pamukkale University, Faculty of Medicine, Department of Histology and Embryology, Denizli, Türkiye

ABSTRACT

This study was aimed to investigate the therapeutic effectiveness of sericin in rats with monosodium iodoacetate (MIA)- induced knee osteoarthritis (KOA), focusing on evaluating its effectiveness via the TGF-β/Smad pathway. The KOA model was established through the injection of MIA into the knee joint, and the rats were randomly allocated into three groups: group 1 (control), group 2 (KOA control), and group 3 (KOA+sericin). Sericin was administered intra-articularly to rats on days 1,7,14, and 21 (0.8 g/kg/mL, 50 µL). After 21 days, the rats were sacrificed, and serum samples were analyzed using the ELISA method to measure transforming growth faktör-Beta (TGF-β1), mother against decapentaplegic homolog 2 (Smad2), and connective tissue growth factor (CTGF) levels. Additionally, knee joint samples underwent histopathological evaluations with hematoxylin-eosin staining and immunohistochemical assessment using TGF-β1 and Smad2/3 antibodies. Serum TGF-β1 and CTGF levels were significantly increased in group 2 vs. group 1 ($P < .05$). A statistically significant decrease was observed in group 3 ($P < .05$). Serum Smad2 levels were not significantly different between groups. Histopathologically, group 2 showed a subchondral bone tissue, degeneration of the cartilage and deep fissures. On the other hand, group 3 showed reduced degeneration in chondrocyte cells, increased cartilage thickness, and a cartilage matrix that appeared close to normal were noted. Immunohistochemically, group 2 exhibited an increase in TGF-β1 and Smad expression, whereas group 3 decreased these expressions than group 2. Sericin demonstrates potential efficacy in the experimental KOA model in rats through the TGF-β1/Smad pathway. Consequently, sericin may emerge as a promising therapeutic agent for the treatment of KOA with further support from advanced clinical trials.

Keywords: Knee osteoarthritis, sericin, TGF-β1/smad pathway

ÖZ

Bu çalışmada; monosodyum iyodoasetat (MIA) ile diz osteoartrit (DOA) modeli oluşturulan sıçanlarda serisinin terapotik etkinliğinin incelenmesi ve bu etkinliğinin TGF-β/Smad yolağı üzerinden değerlendirilmesi amaçlanmıştır. Sıçanlarda DOA modeli oluşturmak için diz eklemine MIA enjekte edilmiş ve ardından sıçanlar rastgele 3 gruba ayrılmıştır (1. grup (kontrol), 2. grup (DOA kontrol), 3. grup (DOA+serisin)). Sıçanlara, serisin 1, 7, 14 ve 21. günlerde (50 µL, 0,8 g/kg/mL) intraartiküler olarak uygulanmıştır. Sıçanlar 21 günün sonunda sakrifiye edilerek elde edilen serum örneklerinde transforming büyüme faktör-Beta (TGF-β1), Smad2 ve bağ doku büyüme faktör (CTGF) seviyeleri ELİSA yöntemi ile belirlenmiştir. Ayrıca diz eklem örneklerinde Hematoksilen-eozin boyası ile histopatolojik, TGF-β1 ve Smad2/3 antikorları ile immünohistokimyasal değerlendirmeleri gerçekleştirilmiştir. Serum TGF-β1 ve CTGF düzeylerinde 2. grupta, 1. gruba göre anlamlı olarak artış tespit edilmiş ($P < ,05$), tedavi verilen 3. grupta ise istatiksel olarak anlamlı azalma görülmüştür ($P < ,05$). Serum Smad2 düzeylerinde gruplar arasında anlamlı fark saptanamamıştır. Histopatolojik olarak 2. grupta subkondral kemik dokusu ve kıkırdak dejenerasyonu ve derin çatlaklar görülmüştür. 3. grupta ise kondrosit hücrelerindeki dejenerasyonun azaldığı kıkırdak dokusunun kalınlığının arttığı ve kıkırdak matriksin normale yakın olduğu izlenmiştir. İmmünohistokimyasal olarak 2. grupta TGF-β1 ve Smad ekspresyonlarında artışlar görülürken, 3. grupta 2. gruba kıyasla bu ekspresyonlarda azalmalar sergilenmiştir. Serisin sıçanlarda deneysel DOA modelinde TGF-β1/Smad yolağı üzerinden potansiyel etkinlik göstermektedir. Sonuç olarak, serisin ileri klinik çalışmalarla desteklenmesiyle DOA tedavisi için umut verici bir terapötik ajan olabilir.

Anahtar Kelimeler: Diz osteoartrit, serisin, TGF-β1/smad yolağı

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Corresponding author/Sorumlu Yazar:
Gülşah GÜNDOĞDU
E-mail: gdemirkaya81@gmail.com

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INTRODUCTION

Osteoarthritis (OA), the most common joint disorder, causes cartilage in the synovial membrane to degenerate, resulting in joint stiffness, pain, limited mobility, local pain, crepitation and varying degrees inflammation. OA affects not only the articular cartilage but also the subchondral bone, capsule, ligaments, synovium and surrounding muscle tissue.[1] The most common form is knee OA (KOA).[2]

Although many risk factors have been implicated in the etiology of OA, the pathophysiological processes are not fully understood. Therefore, it is not possible to talk about a single mechanism explaining the development of OA. OA is a dynamic and metabolically active process in which destruction and repair occur simultaneously due to a combination of biochemical and mechanical factors; however, over time, the balance shifts in favor of destruction.[3,4] As a result of a series of reactions in the articular chondrocytes, abnormal changes occur in the extracellular matrix (ECM) and the homeostasis of the articular cartilage is disrupted.[5] Under stress, chondrocytes release inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), which induce the synthesis of metalloproteinases (MMPs), as well as other inflammatory cytokines and chemokines.[5-7] The inflammatory process in OA involves the release of pro-inflammatory cytokines, proteinases, and mediators such as reactive oxygen species (ROS) from the inflamed area and this mechanism is considered crucial in the development and progression of OA.[8,9]

Transforming growth faktör-Beta (TGF-β) is a fibrogenic factor that plays an important role throughout many processes. These include cell proliferation, migration, apoptosis, differentiation, and stimulation of ECM synthesis.[10] TGF-β binds to receptors on the cell surface, specifically the TGF-β type 1 and 2 receptors. It induces phosphorylated mother against decapentaplegic homolog 2/3 (Smad 2/3) and initiates intracellular signalling. Smads are the only known TGF-β1 receptor substrate. Therefore, Smad/ connective tissue growth factor (CTGF) signalling is essential for TGF-β-induced fibrogenesis.[7] It has been shown that TGF-β has a role to play in all stages of chondrogenesis. TGF-β's inhibitory effect on the terminal differentiation of chondrocytes relies significantly on the pivotal role played by Smad2 and Smad3 as key signaling molecules. CTGF is considered to be an important amplifier of the pro-fibrogenic effect of TGF-β1. It functions as an important down regulator of TGF-β1/Smad signalling in mesenchymal cells and fibroblasts.[7,11]

Several integrins play a significant role in the development and progression of OA by interacting with the ECM and mediating intracellular signalling pathways.[12,13] Integrin-derived growth factors like TGF-β are involved in bone formation and differentiation, with elevated expression observed in OA patients compared to those without OA.[14] Smad proteins mediate TGF-β signalling, impacting chondrocyte differentiation and the PI3K/Akt pathway, contributing to increased cholesterol synthesis in OA.[15]

Because biomaterials can mimic both the biological and mechanical functions of the natural ECM, the selection of appropriate biomaterials for treatment is important.[16] Silk proteins are biomaterials that have become the focus of research in recent years due to their natural occurrence.

The silkworm cocoon (Bombyx mori) consists of two main proteins, sericin and fibroin. The fibroin in the cocoon is a protein bound by disulphide bonds in the form of thin twin filaments and wrapped in successive layers of sticky sericin that form the silk.[17] The sericin formed by hydrolysis of silk proteins have been shown to have various biological activities: antioxidant, anti-diabetic, antitumour, antiviral, antibacterial, hypocholesterolemic, immunoregulatory.[18] Sericin has been found to have a low anti-inflammatory effect by reducing the release of inflammatory cytokines such as TNF-α, [19] while also increasing the production of anti-inflammatory cytokines including TGF-β, IL-4, and IL-10.[17] Sericin, easily accessible, natural, cost-effective, and biocompatible biomaterial. It has positive effects on tissue repair, stimulating the proliferation of fibroblasts and keratinocytes, producing regulatory cytokines essential for the wound healing process, and actively contributing to the synthesis of ECM proteins, playing a critical role in re-epithelialization and overall healing.[20] Sericin also enhances the activity of antioxidant enzymes by scavenging free radicals and ROS.[21,22] Additionally, it is known that sericin enhances the production of anti-inflammatory cytokines such as TGF-β, IL-4, and IL-10 [17], and it regulates the expression of TGF-β1-3 to prevent scar tissue formation during wound healing.[23]

There is a very limited number of studies in the literature investigating the pathogenesis and functional prognosis of experimental OA in more detail. Because of their shortcomings, there is a need to investigate alternative therapies that may shed new light on current treatments and better understand the prognosis of the disease.

Although there are several studies showing the tissue repair/regeneration efficacy of the sericin, there are no studies evaluating its efficacy in the treatment of KOA and the determination of this efficacy via the TGF-β/Smad

pathway. It is also known that the TGF-β/Smad pathway plays an important role in the mechanism of OA. The pathophysiology of OA is not fully understood. Therefore, sericin can also be used in the treatment of KOA, a common disease today. In this context, this study aimed to investigate the effectiveness of sericin in rats with monosodium iodoacetate (MIA)-induced KOA model and assess its efficacy via TGF-β/Smad pathway.

MATERIALS AND METHODS

Animal Model Induction and Experimental Treatments

Animal studies were approved by the Animal Experiments Local Ethics Committee of Pamukkale University (date 09.11.2021, number PAUHADYEK-2021/E-60758568-020-132719/08). Twenty-one female Wistar albino rats (12-14 weeks old, 200-250 g) were purchased from the Medical Experimental Research and Practice Centre of the Pamukkale University. The animals were maintained in a controlled environment at a consistent temperature of 23±2°C, with 50% humidity, and subjected to a regular light-dark cycle (lights on at 8 am, lights off at 8 pm). They were accommodated in specially designed cages and received attentive care under veterinary supervision. MIA (Sigma–Aldrich, Missouri, USA) was used to induce an experimental KOA model in rats. In the literature, MIA is defined as intra-articularly (i.a.) injection is the most commonly used method to experimentally induce KOA in rats.[24] MIA was dissolved in a 0.9% NaCl solution, and for the induction of the experimental KOA rat model, a 30 G needle was utilized to inject a solution of MIA (1.5 mg/50 µL per animal) into the right patella of the rats. A combination of ketamine (Eczacibasi, Parke-Davis, Istanbul, Turkey) and xylazine (Alfasan International BV, Woerden, Holland) general anaesthesia was used for the injection procedure.

One day after the establishment of the experimental KOA model, the rats were randomly selected and were divided into three equal groups to begin the experimental procedure. The experimental groups are shown in Table 1. The study was completed with the indicated number of rats.

Commercially purchased sericin protein (S5201, Sigma–Aldrich, Missouri, USA) was prepared by dissolving in PBS (Sigma–Aldrich, Missouri, USA) (Ph: 8.5).[25]

At the end of all treatments, the rats were fasted overnight. They were allowed free access to water. All rats were sacrificed under i.p. general anaesthesia (ketamine hydrochloride + 2% xylazine hydrochloride).

Table 1: Experimental Groups

Group Name	Experimental Treatments	n
Group 1 (Control)	Non-treatment	7
Group 2 (KOA Control)	A KOA model was induced by a single injection of MIA into the right patellar ligament of rats	7
Group 3 (KOA+Sericin)	Rats with induced KOA were exposed to sericin (0,8 g/kg/mL) through i.a. injection (50 µL per animal) on days 1, 7, 14, and 21	7

Blood samples were collected from the abdominal aorta and subsequently centrifuged at 2000 g for 15 minutes. The resulting serum samples intended for TGF-β1 (E-EL-0162, Elabscience, Texas/ABD), Smad2 (E-EL-R2582, Elabscience, Texas/ABD), and CTGF (E-EL-R0259, Elabscience, Texas/ABD) the solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) analyses were then stored at -80°C until the day of the study.

Knee joints (including patella and articular capsule) of sacrificed rats were removed and placed in 10% formalin tubes for histopathology. Samples were sent to Pamukkale University Faculty of Medicine, Histology and Embryology Laboratory for histopathological analysis using hematoxylin-eosin (H&E) and immunohistochemical analysis using TGF-β1 (sc-130348, Santa Cruz, Texas/USA), Smad2/3 (sc-133098, Santa Cruz, Texas/USA) antibodies.

ELISA Analyses

Measurement of TGF-β1, Smad2, and CTGF Levels

Serum samples were analyzed for the levels of TGF-β1 (E-EL-0162, Elabscience, Texas/ABD), Smad2 (E-EL-R2582, Elabscience, Texas/ABD), and CTGF (E-EL-R0259, Elabscience, Texas/ABD) using ready-to-use measurement kits employing the ELISA method, following the manufacturer's instructions. The obtained results were expressed in ng/mL for TGF-β1 and Smad2, and in pg/mL for CTGF.

Histological and Immunohistochemical Assessment

H&E Staining

For histopathological procedures, knee samples were taken to Pamukkale University Faculty of Medicine, Department of Histology and Embryology Laboratory in labelled bottles containing 10% buffered formaldehyde. The samples were kept in formaldehyde for 72 hours. Routine tissue tracking was performed and 5 micron thick sections were prepared from the paraffin blocks, ready for sectioning with a Leica brand microtome (RM2125RT). The sections were stained with H&E stain. Finally, each slide

was examined under a light microscope (Olympus Bx51 with DP72 camera system).

Immunohistochemical Staining

Tissue blocks were sectioned at 5 µm using a microtome. Immunohistochemistry was performed according to the manufacturer's instructions. Rat TGF-β1 (sc-515284, 1:50 dilution) and Smad 2/3 (Ab 9722, 1:50 dilution) antibodies were used for 60 minutes. A secondary antibody (Abcam HRP/DAB Detection IHC Kit, ab80436) was used according to the kit procedure. The sections were subsequently incubated with 3,3-diaminobenzidine (Dako Cytomation) and counterstained using Mayer's hematoxylin (Dako Cytomation).[26] Afterward, the sections were thoroughly washed with running water. Each of them was kept in 50%, 70%, 80%, 96%, 100% ethyl alcohol series for 2 minutes. Then the tissues were kept in xylene I and xylene II for 2 minutes each. The tissues taken from xylene were covered with entellan without waiting for the tissues to dry and examined in Olympus Bx51 high power light microscope and images were taken. TGF-β1 and Smad2/3 staining localisations were evaluated separately for each rat.

Image Analysis

An Olympus Bx51 high performance light microscope was used to examine the tissue samples. Ten randomly selected fields in each specimen were scored at 40x magnified. The scores were assessed semiquantitatively using light microscopy, analyzing specimens obtained from each rat. The scores were graded as strong staining (++++), moderate staining (+++), weak staining (+) and no staining (-).

Statistical Analysis

As a result of the power analysis, which was performed on the assumption that a large effect size (f=0.7) would be obtained in the study, it was calculated that 80% power with 95% confidence could be obtained if at least 21 rats were used (at least 7 rats for each group). It was decided to start the study with 7 rats per group, for a total of 21 rats.

The collected data were analyzed using SPSS 21.0 (IBM SPSS Statistics 21 software (IBM SPSS Corp., Armonk, NY, USA). Mean±standard deviation was used to express continuous and categorical variables. The normal distribution compatibility of the variables was assessed through the Shapiro-Wilk test. When parametric test assumptions were met, one-way analysis of variance was employed for comparing differences among independent groups. In cases where parametric assumptions were not satisfied, Kruskal-Wallis analysis of variance, with Mann-Whitney U test and Bonferroni correction as a post hoc test, was utilized to assess independent group differences. Statistical significance was considered as $P < .05$.

RESULTS

Determination of Serum TGF-β1, Smad2, and CTGF Levels

TGF-β1 levels were statistically significantly increased in group 2 (3.195 ± 0.53) compared to group 1 (1.927 ± 0.23) ($P = .001$). However, it was statistically significant lower in the group 3 (2.359 ± 0.41) compared with the group 2 ($P = .042$) (Figure 1).

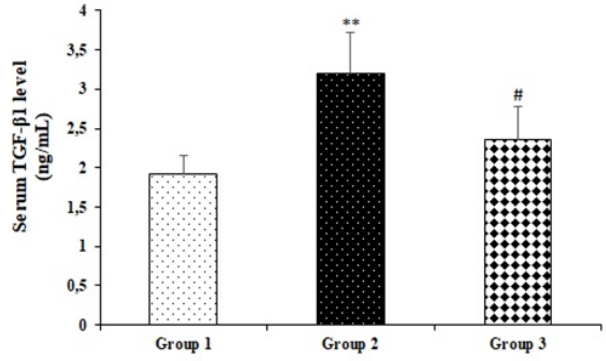

Figure 1. TGF-β1 levels in the experimental groups. (n=7; Results represent mean ± standard deviation; Mann Whitney U-Test was used; **: $P < .001$ vs. group 1; #: $P < .05$ vs. group 2)

Serum Smad2 level increased in the group 2 (0.154 ± 0.05) compared to the group 1 (0.260± 0.03) and decreased in the group 3 compared with the group 2. However, these values did not reach statistically significant level ($P > .05$) (Figure 2).

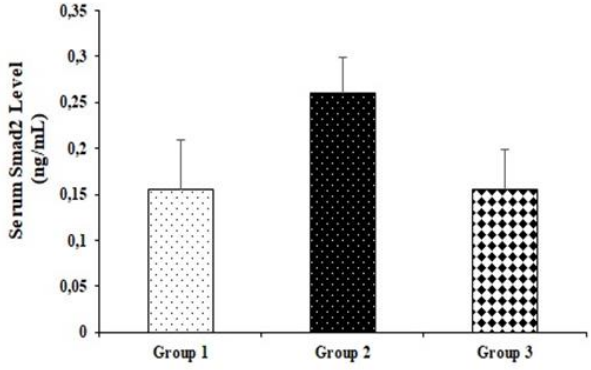

Figure 2. Smad2 levels in the experimental groups. (n=7; Results represent mean ± standard deviation; Mann-Whitney U-Test was used)

There was a statistically significant increase in serum CTGF levels in group 2 (152.42 ± 45.51) compared to group 1 (269.98 ± 41.24) ($P = .003$). However, It was statistically significant lower in the group 3 (159.26 ± 42.92) compared with group 2 ($P = .008$) (Figure 3).

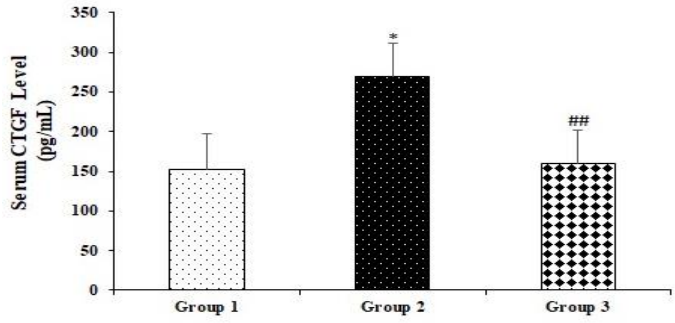

Figure 3. CTGF levels in the experimental groups. (n=7; Results represent mean ± standard deviation; Mann-Whitney U-Test was used; *: $P < .01$ vs. group 1; ##: $P < .01$ vs. group 2)

Histopathological Results

A normal morphology of the joint structure was observed in group 1. Chondrocytes had normal structure and cartilage matrix had normal density in the cartilage tissue. Group 2 had significantly thinner cartilage than group 1. Damage to the articular cartilage surface and structural fractures with reduced chondrocyte numbers were observed. In particular, marked degeneration of some chondrocytes was observed. Degeneration of subchondral bone tissue was also noted. In the treated group 3, the degeneration of chondrocyte cells decreased. The thickness of the cartilage tissue increased and a cartilage matrix close to normal was observed (Figure 4).

Immunohistochemical Results

Table 2 shows the semi-quantitative scoring results obtained from the light microscopic evaluations of the knee joint tissues obtained from the rats.

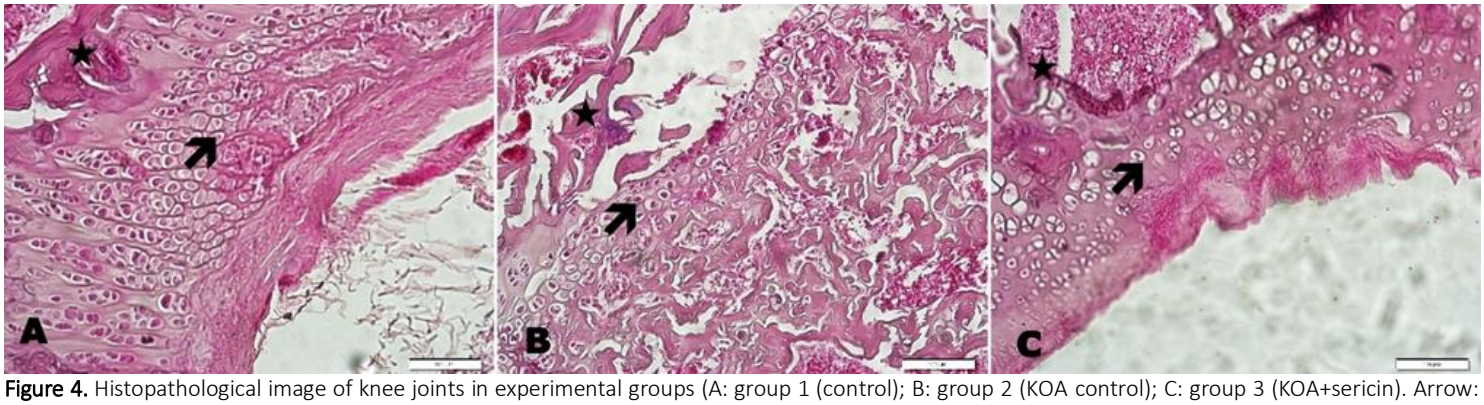

Figure 4. Histopathological image of knee joints in experimental groups (A: group 1 (control); B: group 2 (KOA control); C: group 3 (KOA+sericin). Arrow: cartilage tissue, Star: bone tissue. H&E, x100, Bar;100 µM))

The expressions of TGF-β1 and Smad2/3 in the cartilage and in the bone tissue was negative in groups 1 and 3. However, positive staining in cartilage matrix and chondrocyte membrane was found in group 2. Bone marrow showed intense positive staining in all groups (Figure 5).

Table 2. Semi-quantitative scores obtained from light microscopy of knee joint tissues from rats in the experimental groups

Groups	Group 1			Group 2			Group 3		
	Cartilage tissue	Bone tissue	Bone marrow	Cartilage tissue	Bone tissue	Bone marrow	Cartilage tissue	Bone tissue	Bone marrow
TGFβ1	-	-	+++	++	-	+++	-	-	+++
Smad2/3	-	-	+++	++	-	+++	-	-	+++

DISCUSSION

Sericin's anti-inflammatory properties in various diseases and its activity on the TGF-β1 pathway are well known. [27,28] TGF-β1 plays an important role in the development of OA. It influences chondrocyte differentiation through Smad signaling. Therefore, we hypothesized that the sericin may act through the TGF-β1/Smad pathway in the experimental KOA model. In line with our hypothesis, an experimental model of KOA induced with MIA has been established in this study. The effects of sericin on TGF-β1/Smad were evaluated by ELISA and histological analysis after sericin administration to experimental KOA rats. In present study, sericin was found to be effective on the TGF-β1/Smad pathway, which plays an important role in the pathogenesis of KOA in an experimental KOA model.

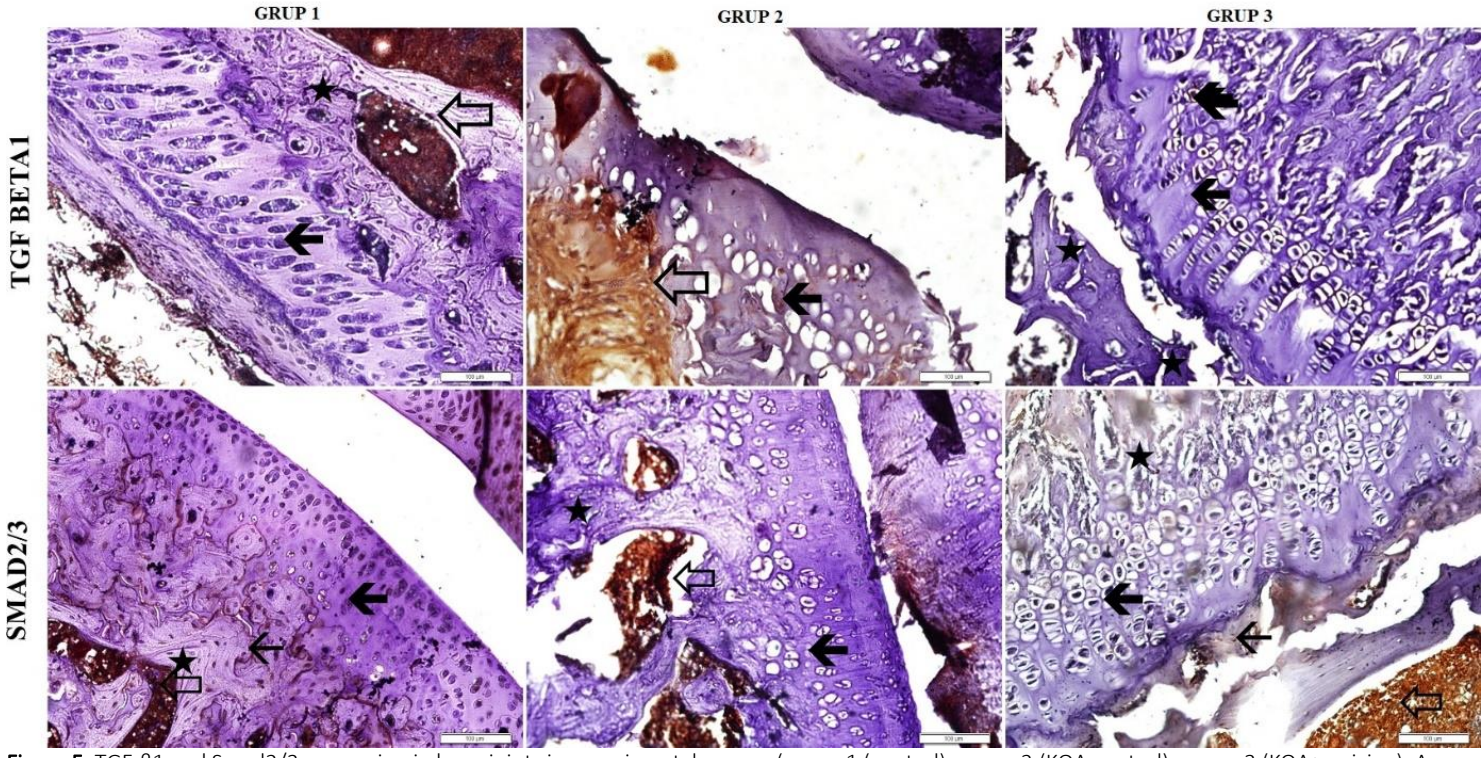

Figure 5. TGF-β1 and Smad2/3 expression in knee joints in experimental groups (group 1 (control); group 2 (KOA control); group 3 (KOA+sericine). Arrow: cartilage tissue, Star: bone tissue, Thin Arrow: Positive expression domains, Thick Arrow: bone marrow, Immunoperoxidase&Hematoxylin x40, Bar; 100µM)

Sericin has also been shown to increase reduced cartilage thickness. It has been determined that it reduces cartilage degeneration and deep cracks.

KOA is the most common joint disease and is characterized by the degeneration of the joint cartilage, which is protected by the synovial membrane.[29] Besides cartilage, OA can affect the subchondral bone, synovial membrane, tendons, capsules and surrounding muscle. OA is a slowly progressive chronic disease affecting the knee.[1] There are several risk factors associated with OA. However, the exact pathophysiological process and basic mechanisms are not fully understood. As articular chondrocytes hypertrophy in OA, the ECM is degraded and articular cartilage fragments form. This is followed by vascular invasion, subchondral bone sclerosis and the formation of osteophytes at the edge of the joint. Progressive degeneration of the articular cartilage is characteristic of OA. This leads to radiographic joint space narrowing, subchondral sclerosis and osteophyte formation.[30]

The TGF-β superfamily plays a crucial role in various biological processes such as growth control, immune response, cell differentiation, early development, and particularly skeletogenesis.[31] TGF-β is considered the main initiator of chondrogenesis, influencing all stages from condensation to terminal differentiation and mainly stimulating cartilage differentiation in early chondrogenesis. Smad signaling regulates chondrocyte terminal differentiation, believed to be significant in OA pathogenesis. However, factors beyond Smad signaling may also influence chondrocyte differentiation and contribute to OA development.[32] Recent research has focused on TGF-β's role in OA, as multiple joint cell types, including cartilage cells, synovial fibroblasts, and macrophages, can produce and release TGF-β. Alterations in the TGF-β pathway and components can disrupt cartilage homeostasis, leading to OA. Studies using mouse models have emphasized TGF-β's importance, showing that Smad3 knockout or conditional deletion causes OA-like degenerative joint disease.[33] Active TGF-β has been found in OA patients' synovial fluid, leading to OA-like changes in the knee joint upon exposure to external TGF-β, which depends on dosage and time.[34] TGF-β activation is believed to enhance cartilage proteoglycan synthesis. However, prolonged exposure or repeated intra-articular application of TGF-β can lead to adverse effects in articular cartilage, such as focal proteoglycan loss and microcracks in the deep cartilage layer. Short-term treatment of chondrocytes with TGF-β reduces MMP-13 levels, whereas prolonged stimulation upregulates MMP-13, mainly through Smad3 and Runx2 mechanisms. This dual role implies that both deficient and excessive activation of TGF-β can contribute to joint pathology in OA.[33,35] The cartilage and bone in joints experience continuous mechanical stress from daily activities, which is crucial for maintaining cartilage homeostasis. However, overuse can lead to joint damage, altering biomechanical properties seen in conditions like OA.[36] Mechanotransduction and mechanical responses have been shown to interact with TGF-β

signalling, although the mechanisms remain incompletely understood. TGF-β / Smad2/3 signalling affected by mechanical forces.[37] TGF-β plays a role not only in regulating chondrocyte behavior and cartilage degradation but also in osteophyte formation, a hallmark of OA. Osteophytes in experimental OA show strong expression of TGF-β1 and Smad2/3. TGF-β is a multifunctional cytokine involved in inflammation and immunity. During OA development or associated inflammation, damaged joint tissue releases TGF-β. TGF-β1 expression increases in subchondral bone in OA, both in human and mouse models. In summary, it is evident that TGF-β plays a role not only in cartilage degradation but also in new cartilage and bone formation, such as in osteophytes.[32]

There are two main proteins in the silk cocoon, sericin and fibroin. Fibroin is found in the cocoon. It is a protein wrapped in layers of sericin.[17] Sericin, formed by the hydrolysis of silk proteins, has several biological activities such as anti-diabetic, hypocholesterolemic, anti-oxidant, immunoregulatory, anti-tumor, anti-viral, anti-bacterial.[18] Panilaitis and colleagues investigated the inflammatory potential of silk fibers and extracts in vitro and found an increase in the release of TNF-α.[19] Another study showed that sericin increases anti-inflammatory cytokines such as IL-4 and IL-10 production.[17] According to the study conducted by Qi et al., sericin was reported to exhibit anti-inflammatory effects in the wound healing process. It was observed to promote angiogenesis and prevent the formation of scar tissue by regulating the expression of TGF-β1-3.[23] Sericin was shown to attenuate glomerulosclerosis and renal interstitial fibrosis by blocking activation of the TGF-β1/Smad3 pathway in rats with diabetic nephropathy, and to protect and prevent renal damage in rats with diabetic nephropathy by Song et al.[38] Our previous study demonstrated the efficacy of sericin in the treatment of experimental Achilles tendonopathy in rats via the TGF-β1/Smad signalling pathway.[39]

In this study, we evaluated the effects of sericin on the KOA model through the TGF-β1/Smad signaling pathway. TGF-β1, Smad2 and CTGF levels, which are important in the pathogenesis of OA, were determined by ELISA in serum samples from rats (Figure 1-3). It was found that TGF-β1, Smad2 and CTGF levels increased in the group 2 compared to the group 1. These levels decreased with sericin treatment (group 3). However, only Smad2 did not achieve statistically significant levels. In addition, TGF-β1 and Smad2/3 expressions were evaluated by IHC staining (Figure 5). While group 2 showed increased immunostaining, the group 3 showed immunostaining similar to the group 1.

In addition, in this study, knee joint samples from all groups were histopathologically examined using H&E staining to demonstrate the efficacy of sericin in the treatment of KOA. In group 2, thinning of the cartilage tissue, damage to the articular cartilage surface and structural fractures were observed, as well as a decrease in the number of chondrocytes and degeneration. Sericin treatment was shown to decrease chondrocyte degeneration, increase chondral thickness, and restore near-normal chondral matrix (Figure 4).

Sericin is known to have a positive effect on tissue damage repair, keratinocyte and fibroblast growth and wound healing.[20] It has also been suggested that TGF-β plays a central role in cartilage destruction, osteophytosis and synovial fibrosis in the development of OA.[20] This means that sericin can also be used to treat KOA, which is now a common disease.

In this context, this study, which focused on the effects of sericin treatment on KOA through the TGF-β1/Smad signalling pathway, demonstrated its efficacy. Biochemical and immunohistochemical analysis showed that sericin effectively reduced TGF-β1/Smad signaling, a key pathway for tissue repair in KOA. In addition, histopathological evaluations of sericin showed that it increased cartilage tissue and reduced degeneration. These findings suggest that because of its beneficial effects on inflammation and tissue repair, sericin may be a promising therapeutic agent for the treatment of KOA.

Ethics Committee Approval: This study was approved by the Animal Experiments Local Ethics Committee of Pamukkale University Medical Faculty (Ethics Committee approval date 09.11.2021, decision no: PAUHADYEK-2021/E-60758568-020-132719/08).
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Experimental Velogenic Viscerotropic Newcastle Disease Virus Infection in Chickens Immunologically Impaired by Treatment with Cyclophosphamide

Siklofosfamid Tedavisiyle Bağışıklık Sistemi Bozulmuş Erkek Civcivlerde Deneysel Velojenik Visserotropik Newcastle Hastalığı Virüsü Enfeksiyonu

Simeon Chibuko OKAFOR[1]
John Ikechukwu IHEDIOHA[1]
John Osita Arinze OKOYE[1]

[1]University of Nigeria, Faculty of Veterinary Medicine, Department of Veterinary Pathology and Microbiology, Nsukka, Nigeria

ABSTRACT

This study investigated whether lymphocytic depletion following chemical bursectomy influenced the severity of infection and development of lesions in chickens challenged with velogenic viscerotropic Newcastle disease virus (vvNDV). Cockerel chickens treated with cyclophosphamide on days 2, 3 and 4 post-hatch showed loss of weight, atrophy and lymphocytic depletion in the bursa of Fabricius and spleen. At 6 weeks of age, the chickens were assigned to four groups- Bursectomized intramuscularly vvNDV inoculated (BI), bursectomized uninfected (BU), non-bursectomized infected (NBI) and non-bursectomized uninfected (NBU) chickens. The BI and NBI chickens showed significant ($P < .05$) loss of weight than their uninfected controls. Depression, anorexia, greenish diarrhea, listlessness, tremor, and oculo-nasal discharges were observed in both infected groups, but were more severe and frequent in the NBI than in the BI chickens. Total mortalities were 100% and 95.5% for the NBI and BI chickens, respectively ($P > .05$). Lesions in both infected groups included atrophy of the bursa, spleen and thymus. Hemorrhages in the proventricular mucosa, intestines and cecal tonsils, as well as congestion and enlargement of the kidneys were significantly ($P < .05$) more severe and frequent in NBI than BI chickens. Histopathology showed necrosis and depletion of lymphocytes in the three lymphoid organs in both infected groups with more severity in the NBI than BI chickens. These results show that depletion of lymphocytes by treatment with cyclophosphamide may influence the severity of infection and development of lesions in vvNDV infection in cockerel chickens.

Keywords: Bursectomy, cockerel chickens, lymphoid organs, pathogenesis, velogenic Newcastle disease virus

ÖZ

Bu çalışmada, kimyasal bursektomiyi takiben lenfositik tükenmenin, velojenik viskerotropik Newcastle hastalığı virüsü (vvNDV) ile enfekte erkek civcivlerde enfeksiyonun şiddetini ve lezyonların gelişimini etkileyip etkilemediği araştırılmıştır. Kuluçkadan sonraki 2, 3 ve 4. günlerde siklofosfamid ile tedavi edilen erkek civcivlerde kilo kaybı ve bursa Fabricius ve dalakta atrofi ve lenfositik tükenme görülmüştür. Erkek civcivler 6 haftalıkken dört gruba ayrılmıştır: Bursektomize kas içi vvNDV aşılanmış (BI), bursektomize enfekte olmamış (BU), bursektomize enfekte olmamış NBI) ve bursektomize enfekte olmamışlar (NBU). BI ve NBI erkek civcivleri, enfekte olmamış kontrollerine kıyasla önemli ölçüde ($P < .05$) kilo kaybı göstermiştir. Depresyon, anoreksi, yeşilimsi ishal, halsizlik, titreme ve okülo-nazal akıntılar her iki enfekte grupta da gözlenmiş, ancak (NBI'da BI erkek civcivlerine göre daha şiddetli ve sık görülmüştür. Toplam ölüm oranları NBI ve BI erkek civcivleri için sırasıyla %100 ve %95,5'tir ($P > .05$). Her iki enfekte gruptaki lezyonlar arasında bursa, dalak ve timus atrofisi yer almıştır. Proventriküler mukoza, bağırsaklar ve çekal tonsillerdeki kanamaların yanı sıra böbreklerdeki tıkanıklık ve genişleme NBI erkek civcivlerinde, BI erkek civcivlerine göre önemli ölçüde ($P < ,05$) daha şiddetli ve yaygındı. Histopatoloji, her iki enfekte grupta da üç lenfoid organda nekroz ve lenfositlerin tükendiğini, NBI'da BI erkek civcivlerine göre daha şiddetli olduğunu göstermiştir. Bu sonuçlar, siklofosfamid tedavisi ile lenfositlerin tükenmesinin, erkek civcivlerde vvNDV enfeksiyonun şiddetini ve lezyonların gelişimini etkileyebileceğini göstermektedir.

Anahtar Kelimeler: Bursektomi, erkek civcivler, lenfoid organlar, patogenez, velojenik Newcastle hastalığı virüsü

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Corresponding author/Sorumlu Yazar:
Simeon Chibuko OKAFOR
E-mail: simeon.okafor@unn.edu.ng

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INTRODUCTION

Newcastle disease (ND) is a very important disease of poultry, cage and wild birds worldwide. It is caused by the pathogenic strains of Newcastle disease virus (NDV) which is an Orthoavulavirus 1.[1] It is a non-segmented, single-stranded, negative-sense RNA virus belonging to the genus *Orthoavulavirus* 1 subfamily *Avulavirinae* within the family *Paramyxoviridae* and order *Mononegavirales*.[2,3] The virus infects almost all avian species of various ages with adverse economic consequences.[4,5] NDV is a pleomorphic, single stranded RNA virus.[6] The disease is one of the reportable diseases to the World Organization for Animal Health (OIE), because of the adverse economic implications of outbreaks of virulent ND in commercial poultry farms.[7] Control measures such as vaccination represent a huge drain in the economy even in developed nations with well-established poultry industries. Newcastle disease is enzootic in Africa including Nigeria, Asia, Middle East and some countries of Central and South America.[8-11] In recent years vaccination and biosecurity have failed in the control of ND due to the emergence of new strains of velogenic NDV (vNDV) which have very wide antigenic and genetic variation.[12,13] These new strains cause frequent outbreaks of ND in well vaccinated flocks in the farms with resultant great losses to the economy.[8-11] This underscores the need for further research on this disease with a view to deeper understanding of the pathophysiology, pathogenesis and the dynamics of the infection, particularly in poultry.[14-16]

Natural infection is through the oral, ocular and respiratory routes, and upon the invasion of intestinal or tracheal mucosa, the organisms are spread systematically, and carried to organs rich in reticuloendothelial tissues through the blood and lymphatics.[17] The course and severity of the disease can be influenced by the host (species, age, and immune status), virus (strain, pathotype, concentration and route of infection), concurrent infection, stress and environmental factors.[18]

The clinical signs and lesions of ND affect the digestive, respiratory, nervous, reproductive and lymphoid systems, resembling those of other poultry diseases, especially infectious bursal disease (IBD). This makes early diagnosis of the disease difficult in the field. Previous reports had it that IBD cannot establish in young chickens that have undergone bursectomy and in older chickens with partial or complete regression of the bursa, because the B-lymphocytes are the targets cells for IBDV infection.[19,20] Earlier study reported that lymphoid organs suffer severe atrophy in velogenic ND of chickens, as a result of necrosis and depletion of lymphocytes.[21] There is, therefore, the curiosity to find out if the lymphocytes play any role in the establishment and severity of vvNDV infection in chickens as they do prominently in IBDV infection in chickens.

Cyclophosphamide, a tumoricidal agent, has been employed in chemical bursectomy and is known to inhibit the functions of the Bursa of Fabricius, especially when large doses are administered.[20,22-24] According to these reports, cyclophosphamide selectively suppresses the bursa-dependent functions by destroying only the lymphoid cells leaving the bursal reticulum intact, causes a momentary involution of the thymus, and destroys the bursa-dependent tissues and cells in the spleen and cecal tonsils. In this project, we studied the effects chemical bursectomy had in the pathogenesis of velogenic viscerotropic NDV (vvNDV) infection because of the very severe necrosis and depletion of the lymphocytes it causes in the lymphoid organs.

MATERIALS AND METHODS

Chickens

One hundred, day-old cockerel chickens (*Gallus gallus domesticus*) used for this experiment were purchased from a reputable indigenous hatchery. Brooding and rearing were done in isolation on deep litter with provision of feed and water *ad libitum*, and they were not vaccinated against any disease. The chicks were housed, under strict biosecurity measures, in the Poultry Experimental Unit of the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Bursectomy by Cyclophosphamide Treatment

Cyclophosphamide (Endoxan®, Frankfurt, Germany) was procured in a dry state and an aqueous solution was prepared each day by dissolving 250mg in 10ml of distilled water. The cockerel chickens were assigned into two groups of fifty (50) cockerels each. One group of 50 cockerels received 5mg each of cyclophosphamide in 0.2ml of distilled water in the breast muscle on days 2, 3 and 4 of age. These constituted the bursectomized (B) group. The second group of 50 cockerels received only 0.2ml of distilled water each IM as placebo and constituted non-bursectomized (NB) group. At day 18 post-bursectomy (PB), 3 chickens from the B group and 3 from NB group were sacrificed and the efficacy of the bursectomy assessed by observing grossly the lymphoid organs particularly the bursa of Fabricius, the spleen and the thymus. The organs were fixed in 10% formal saline for histopathology.

The NDV Inoculum

A Nigerian strain vvNDV known as duck/Nigeria/903/KUDU–113/1992 was used. It was isolated from apparently healthy ducks, purified and characterized by Echeonwu et al.[25] The strain belongs to NDV class II, genotype XVII.[26] The inoculum had a median embryo effective dose (EID_{50}) of $10^{6.4}$ /ml.

ND Virus Challenge

Hemagglutination inhibition (HI) test was used to certify that the chickens were serologically negative for NDV antibodies at the age of 6 weeks and the chickens were assigned into four experimental groups.

Group 1 comprised 25 bursectomized and vNDV-challenged chickens (BI).
Group 2 comprised 22 bursectomized and unchallenged chickens (BU).
Group 3 consisted of 25 non-bursectomized and vNDV-challenged chickens (NBI).
Group 4 comprised 22 non-bursectomized and unchallenged chickens (NBU).

Each cockerel chicken in groups 1 and 3 received intramuscularly (I/M) 0.2 ml of the NDV inoculum, whereas each cockerel chicken in groups 2 and 4 was given 0.2 ml of phosphate buffered saline (PBS) via the same route as placebo.

The four experimental groups were housed separately.

Clinical Manifestations

Observations were made twice daily for clinical signs in all the groups, following vNDV challenge. Records of both morbidity and mortality were also taken. Ten (10) chickens from each group were randomly selected and weighed on days 0, 3 and 6 post-infection (PI) and the mean weight and percentage weight loss calculated and recorded.

Observation for Pathological Changes

Dead chickens from the infected groups and those sacrificed in the uninfected groups were necropsied on days 4, 5, 6, 7, 8 and 9 PI. Lesions of the gastrointestinal tracts and the kidneys of each chicken were studied, scored and recorded thus: no lesion = 0, mild lesion = 1, moderate lesion = 2 and severe lesion = 3.

Histopathology

Samples were collected from the bursa of Fabricius, spleen and thymus and fixed in 10% formal saline for 48h. The fixed organs were routinely processed and sectioned at 5µm thickness after fixation, and stained with hematoxylin and eosin.[7] The slides were viewed under a light microscope and then photographed with digital camera.

Virus Isolation

Samples of the Bursa of Fabricius, spleen, thymus, and intestine were aseptically collected on day 5 PI from 3 recently dead chickens in each group. The samples were refrigerated at −20°C until they were used for virus isolation in embryonated chicken eggs following the method of World Organization for Animal Health (OIE).[7]

Statistical Analysis

The mean body weight and significance of the differences were analyzed using one-way analysis of variance (ANOVA). Fisher's exact test and Sample t-test were used to analyze the mortality data and the gross lesions, respectively. Variant and significant means were separated *post hoc* using the least significant difference method, and using t-test for Equality of means, respectively.[27] The level of significance was accepted at $P < .05$.

The procedures followed in this investigation have been approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria (Approval date: 10.03.2020, Number: FVM-UNN-IACUC-0340), and care was taken to minimize the number of animals used.

RESULTS

Effect of Bursectomy on the Chickens

The bursectomized chicken showed weight loss, and severe and moderate atrophy of the bursa of Fabricius and spleen, respectively on day 18 post bursectomy (PB) (Figures 1-A, B). The thymus, however, did not show any obvious change in size in the bursectomized chickens compared to the non-bursectomized group (Figure 1-C). Histopathological examination of the bursa of cyclophosphamide treated chickens showed severe necrosis and depletion of lymphocytes, whereas the untreated chickens had normal bursa (Figures 2-A, B). The spleen of bursectomized chickens showed moderate necrosis and depletion of lymphocyte, while non-bursectomized chickens were normal (Figures 2-C, D). The thymus of cyclophosphamide treated chickens did not show necrosis and lymphocytic depletion, likewise the untreated group (Figures 2-E, F).

Clinical Signs

There were no clinical manifestations in the BU and NBU chickens. Clinical signs were first observed in NBI and BI chickens on days 2 and 3 PI, respectively. By day 3 PI, 60%

Figure 1. Bursa of Fabricius, spleen, thymus. Treatment with cyclophosphamide. **Figure 1-A**. The bursa of chickens treated with cyclophosphamide showed severe atrophy (B or bursectomized), whereas, the untreated group had normal bursa (NB or non-bursectomized). **Figure 1-B**. The spleen of the treated chickens showed moderate atrophy (B), while those of the untreated (NB) group were normal. **Figure 1-C.** There was no clear difference between the thymus of the treated (B) and those of the untreated (NB) chickens.

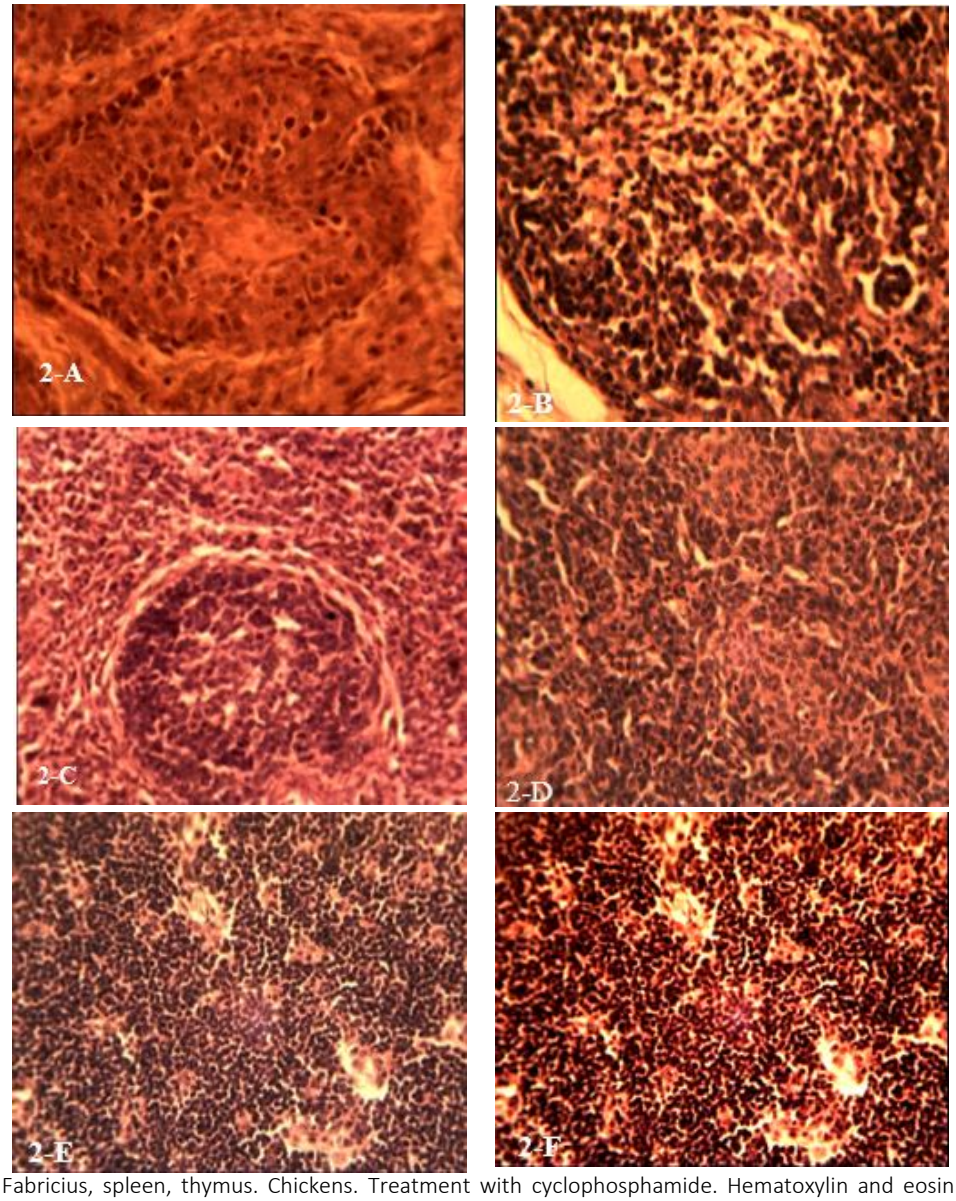

Figure 2. Bursa of Fabricius, spleen, thymus. Chickens. Treatment with cyclophosphamide. Hematoxylin and eosin x 400. Day 18 post bursectomy (PB). **Figure 2-A.** Bursa. Bursectomized uninfected (BU) chicken showing severe lymphocytic depletion. BF contains 100% B-lymphocytes. **Figure 2-B.** Bursa. Non-bursectomized uninfected (NBU) chicken fully populated by B-lymphocytes. **Figure 2-C**. Spleen. Bursectomized uninfected (BU) chicken showing moderate lymphocytic depletion on day 18 PB. Spleen contains 50% B-lymphocytes located in the GF and PALS. **Figure 2-D**. Spleen. Non-bursectomized uninfected (NBU) chicken with normal spleen. **Figure 2-E**. Thymus. Bursectomized uninfected (BU) chicken fully populated by T-lymphocytes. Thymus is made up of almost 100% T-lymphocyte and bursectomy affects only the B-lymphocytes. **Figure 2-F**. Thymus. Non-bursectomized uninfected (NBU) chicken. Normal thymus with full lymphocyte population.

of BI group and 75% of NBI group showed loss of appetite, ruffled feathers and severe depression. 0% of BI and 5% NBI chickens were paralyzed while 2% of BI and 30% NBI chickens showed opisthotonus and muscle twitching and some had soiled vents with whitish to greenish diarrhea. By day 4 PI, 75.7% of BI and 100% NBI chickens showed marked depression while 2.7% of BI and 5.4% NBI chickens died. By day 5 PI, 72.2% of BI and 100% NBI chickens were depressed while 47.2% of BI and 51.4% NBI chickens died. By day 6 PI, 94.7% of BI and 93.8% NBI chickens suffered depression while mortality of 47.4% and 56.3% was recorded in BI and NBI chickens respectively. A significant (P < .05) weight loss was observed in both BI and NBI chickens compared to their respective uninfected controls at day 6 PI (Figure 3). The percentage weight loss was 12.55% and 24.48% in the BI and NBI chickens respectively. At day 7 PI, 90% of BI and 85.7% NBI chickens suffered depression while mortality of 40% and 42.9% was recorded in BI and NBI chickens respectively. By day 8 PI, 66.7% of BI and 100% NBI chickens showed depression with mortality of 50% and 75% in BI and NBI chickens respectively. At day 9 PI, 66.7% of BI and 100% NBI chickens were depressed with mortality of 66.7% and 100% recorded in BI and NBI chickens respectively. By day 10 PI, the total mortalities were 95.5 and 100 % in BI and NBI respectively. There was, however, no significant difference between the overall mortalities of the infected groups (P > .05) (Table 1).

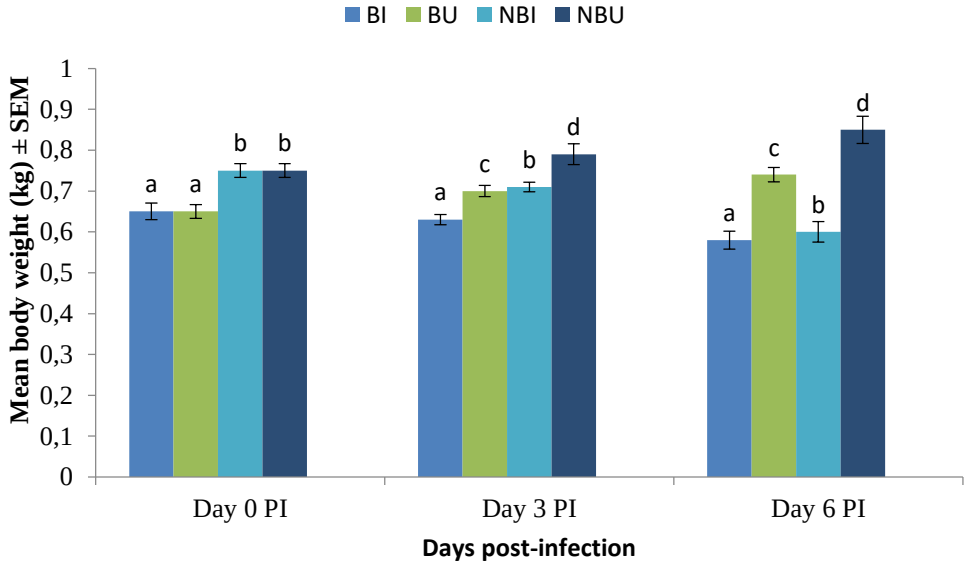

Figure 3: Body weight of chickens in all the experimental groups on days 0, 3, 6 post infection (PI). Weights of groups with different superscripts are statistically significant (P < .05). BI is the bursectomized vNDV infected chickens. BU is the bursectomized uninfected chickens. NBI is the non-bursectomized vNDV infected chickens. NBU is the non-bursectomized uninfected chickens.

Table 1. Comparison of the mortality rate of vvNDV BI and NBI chickens.

	Mortality Rate				
Days PI	BU	BI	NBU	NBI	Statistics
4	0/19	1/22	0/19	2/22	P = .091;(P > .05)
5	0/17[a]	10/21[b]	0/17[a]	12/20[c]	P = .001;(P < .05)
6	0/17[a]	5/11[b]	0/17[a]	5/8[c]	P = .013;(P < .05)
7	0/17[a]	2/6[b]	0/17[a]	1/3[c]	P = .026;(P < .05)
8	0/17	2/4	0/17	1/2	P = .091;(P > .05)
9	0/17	1/2	0/17	1/1	P = .091;(P > .05)

[a,b,c]Different alphabetical superscripts in a row indicate significant differences in the mortalities (P < .05). BU: Bursectomized uninfected; BI: Bursectomized infected; NBU: Bursectomized uninfected; NBI: Non-bursectomized infected.

Gross Lesions

There were no gross lesions in the BU and NBU chickens. The gross lesions in the BI and NBI were congestion of the skeletal muscles, hemorrhages in the proventricular glands, and hemorrhagic ulcers in the intestines and cecal tonsils. Bursa, spleen and thymus were atrophic. The kidneys were swollen and hemorrhagic. The proventricular, cecal and intestinal lesions were more frequent and severe

in the NBI than BI chickens. The scores of the gastrointestinal and kidney lesions were significantly ($P <$ .05) higher in the NBI than BI chickens (Table 2).

Table 2. Comparison of gross lesion scores of vvNDV BI and NBI chickens on day 5 PI.

	Proventricular haemorrhage		Congestion of breast and thigh muscles		Intestinal hemorrhage and ulcer		Congestion and enlargement of kidney	
S/NO	BI	NBI	BI	NBI	BI	NBI	BI	NBI
1	0	2	1	3	0	2	0	0
2	1	2	2	3	0	2	0	1
3	1	3	2	3	1	3	0	0
4	1	2	2	2	1	3	0	0
5	1	2	1	2	1	3	0	1
6	1	3	1	3	0	3	0	1
7	2	3	1	3	0	2	0	0
8	1	3	1	2	0	2	0	1
9	1	3	2	3	0	3	0	0
10	2	2	2	3	1	2	0	1
Mean scores:	1.1 ± 0.18^{a}	2.5 ± 0.17^{b}	1.5 ± 0.16^{a}	2.7 ± 0.15^{b}	0.4 ± 0.16^{a}	2.5 ± 0.17^{b}	0.0 ± 0.00^{a}	0.5 ± 0.17^{b}

[a,b]Different alphabetical superscripts in a row indicate significant difference ($P < .05$) between the mean scores of the lesions in the BI and NBI chickens. BI: Bursectomized infected chickens; NBI: Non-bursectomized infected chickens. Scores: No lesion = 0, mild lesion = 1, moderate lesion = 2, severe lesion = 3.

Histopathology

Histopathology showed congestion, ballooning degeneration, necrosis, depletion of lymphocytes and fibrin deposition in the bursa, spleen and thymus with NBI chickens demonstrating more severe lesions than the BI group (Figure 4-A-F).

Virus Isolation

No virus was isolated from organs of BU and NBU chickens, whereas, there was virus isolation in the BI and NBI chickens on day 5 PI (Table 3). There is hemagglutination activity shown by harvested allantoic fluids with washed chicken red blood cells, in positive cases. A known specific NDV antiserum neutralizes this hemagglutination.

Table 3. Virus isolation in selected organs of the BI and NBI chickens on day 5 PI

Organs	HI Activities
Bursa of Fabricius	+
Thymus	+
Spleen	+
Intestine	+

+ = Virus isolated from the organs.

DISCUSSION

The lesions of vvNDV infection are quite similar to the lesions observed in very virulent infectious bursal disease virus (vvIBDV) infection of chickens. Lesions such as congestion of the skeletal muscles, hemorrhages in the proventricular mucosa, hemorrhagic ulcers in the cecal tonsil, enteritis, severe lymphocytic necrosis and depletion in the lymphoid organs resulting in atrophy of the bursa, spleen and thymus, swollen and hemorrhagic kidneys occur in the two diseases.[28-30] Unarguably, the two diseases are not always easy to differentiate in the field in chickens that have some sub-optimal levels of immunity because they do not show all the clinical signs and lesions. Earlier researchers reported that IBD cannot establish in young chickens that have undergone bursectomy and in older chickens with partial or complete regression of the bursa, because the B-lymphocytes are the targets cells for IBDV infection.[19,20] Evidence that vvNDV infection is also immunosuppressive like vvIBDV infection is gradually emerging as report had it that experimental vvNDV infection suppressed HI antibody response to LaSota vaccination in surviving chickens.[29] These form the basis for this research, as efforts aimed at fully understanding the pathophysiology and pathogenesis of IBD and vvND will assist clinicians in proper and accurate diagnosis.

The severe and moderate atrophy of the bursa and spleen respectively, of cockerel chickens treated with cyclophosphamide, as a result of necrosis and depletion of lymphocytes, which were observed in this study are consistent with previous reports.[20,31,32] This may be supportive of the previous reports that cyclophosphamide selectively suppresses the bursa-dependent functions by destroying only the lymphoid cells leaving the bursal reticulum intact, causes a momentary involution of the thymus, and destroys the bursa-dependent tissues and cells in the spleen and cecal tonsils.[20,24] This may also explain the severe and moderate atrophy of the bursa and spleen, respectively, with no change in the thymus post-cyclophosphamide treatment in this study, as bursa and spleen were reported to be made up of 100% and about 50% B-lymphocytes respectively, and the thymus harbors 100% T-lymphocytes.[20]

Earlier studies have used cyclophosphamide treatment to specifically suppress B-cell dependent humoral immunity to ascertain the role of B and T-lymphocytes in immune responses to infectious pathogens.[20,33]

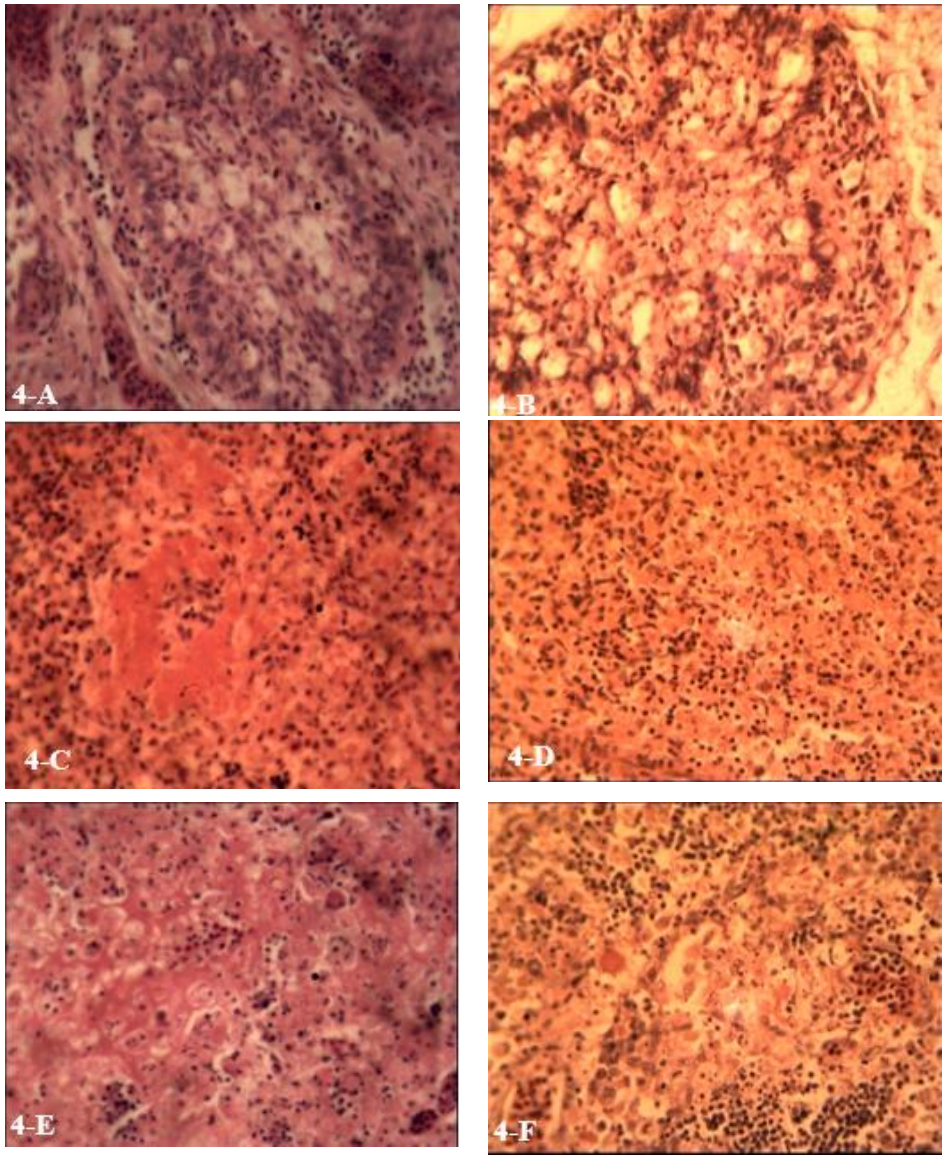

Figure 4. Bursa of Fabricius, spleen, thymus. Chickens. Challenge with vvNDV. Day 5 post infection (PI). Hematoxylin and eosin x 400. **Figure 4-A.** Bursa. Non-bursectomized infected (NB1) chicken showing severe lymphocytic depletion and ballooning degeneration. **Figure 4-B.** Bursa. Bursectomized infected (BI) chicken has severe lymphocytic depletion and microcavities. **Figure 4-C**. Spleen. Non-bursectomized infected (NBI) chicken showing severe lymphocytic depletion and fibrin deposition. **Figure 4-D**. Spleen. Bursectomized infected (BI) chicken showing severe lymphocytic depletion and necrosis. **Figure 4-E**. Thymus. Non-bursectomized infected (NBI) chicken showing congestion, severe necrosis of lymphocytes and deposition of fibrin and ballooning degeneration. **Figure 4-F**. Thymus. Bursectomized infected (BI) chicken showing severe lymphocytic depletion, necrosis and fibrin deposition.

Infections of both BI and NBI chickens produced severe systemic illness, with marked clinical signs of depression, coma lethargy, whitish-greenish diarrhea, reduced water and feed intake, and substantial death by day 6 PI. Similar clinical signs have been observed by other researchers in chickens infected with vvNDV.[21,34-36] These signs, however, were less severe in BI than NBI chickens. This could be explained by the fact that lymphocytes are the only cells that show necrosis in vND and the fact that there was lymphocytic depletion in BI chickens must have caused the reduction in clinical signs in BI chickens compared to the NBI chickens.

In this study, the gross lesions in NBI chickens included congestion of the skeletal muscles, enlargement and atrophy of the lymphoid organs, and ulcerations of the gastrointestinal tracts, the most striking lesions being sharply-demarcated hemorrhagic intestinal ulcers, cecal tonsils and proventricular hemorrhages especially in the dead ones on day 5 PI. Similar lesions in chickens were reported in lymphoid and other organs by previous researchers.[34,37-39] Gastrointestinal tract lesions were preferably scored, as previous report opined that these lesions were suspected to account for high mortalities found mostly in vvNDV infection of chickens.[40] The ulceration of the intestinal mucosa may be due to active viral replication in the intestinal lymphoid follicles. However, intestinal lesions were almost absent in the BI chickens. This could be as a result of depopulation of lymphocytes in the lymphoid follicles of the gastrointestinal tract of BI chickens. In this study, proventricular hemorrhages were found in both BI and NBI

chickens, but the severity was more in the NBI than in the BI chickens as NBI chickens scored higher. There was marked atrophy of the Bursa of Fabricius in both BI and NBI chickens. The atrophy, lymphocyte necrosis and depletion in the lymphoid organs of the infected chickens are consistent with the lesions described for vvNDV infections in domestic poultry.[35,41,42]

Suppression of the immune response has been reported to have important effects on both the pathogenicity of infecting NDV strains and the potential levels achieved by vaccination.[18] Cyclophosphamide is an immunosuppressant and must have contributed to the development of clinical NDV despite lymphocytic depletion which was supposed to prevent development of clinical NDV. Evidence of multifocal and diffused regeneration in bursa and spleen respectively on days 19 and 20 post-cyclophosphamide administration has also been reported.[32] Earlier reports opined that immunohistochemical labeling in NDV infection was confined to large mononuclear cells, and vvNDV replicated in macrophages.[43,44] The B- and T- lymphocytes pass the description of large mononuclear cells, hence supportive of our findings. Hemorrhagic lesions were more frequent and severe in the proventricular mucosa, intestines and ceca in NBI than the BI probably because the bursectomy depleted the lymphocytic populations at those locations. Lymphocytic depletion following chemical bursectomy may, therefore, influence the severity of infection and development of lesions in vvNDV infection in cockerel chickens, suggesting that vvNDV may require both B- and T- lymphocytes to establish in infected chickens.

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Pelvis Anatomy and Morphometric Analysis in New Zealand Rabbits

Yeni Zelanda Tavşanlarında Pelvis Anatomisi ve Morfometrik Analizi

Muhammet Lütfi SELÇUK[1]
Nimet TURGUT[2]

[1]Karamanoğlu Mehmetbey University, Faculty of Health Sciences, Department of Physiotherapy and Rehabilitation, Karaman, Türkiye
[2]Selçuk University, Faculty of Veterinary Medicine, Department of Anatomy, Konya, Türkiye

ABSTRACT

This study aims to obtain three-dimensional models of the cavum pelvis in New Zealand rabbits of both genders using CT images, to measure the pelvis diameters and angles through the created digital models, and to compare female and male New Zealand Rabbits in terms of sexual dimorphism. A total of 20 New Zealand rabbits, 10 females and 10 males, were used in this study. Computed tomography (CT) images of the animals were taken, the images were reconstructed with the MIMICS 20.1 program, and a three-dimensional model of the pelvic cavity was obtained from the two-dimensional images. Morphometric data were obtained by making diameter and angle measurements on the resulting 3D model. Then, the rabbits were dissected and the os coxae was exposed and the anatomical formations were named. When pelvimetry measurements in female and male rabbits were compared, it was seen that all values except pelvic tilt were higher in females. The data reveal that there is no significant difference in the volume and surface area of the right and left os coxae between male and female rabbits ($P > .05$). In this study comparing the morphometric differences of the pelvis in female and male New Zealand rabbits, volume and surface area data were shared for the first time. The collected data could be used for sex discrimination in rabbits, assist physicians in diagnosing patients, serve as a reference for clinical practices, and form the basis for new research.

Keywords: 3D modelling, computed tomography, New Zealand rabbit, pelvic cavity, pelvic bones

ÖZ

Bu çalışmanın amacı, Yeni Zelanda tavşanlarının BT görüntüleri kullanarak her iki cinsiyetteki cavum pelvis'in üç boyutlu modellerini elde etmek, oluşturulan dijital modeller üzerinde pelvis çaplarını ve açı ölçümlerini gerçekleştirerek, dişi ve erkek Yeni Zelanda tavşanlarını cinsel dimorfizm açısından karşılaştırmaktır. Çalışmada 10'u dişi, 10'u erkek olmak üzere toplam 20 adet Yeni Zelanda tavşanı kullanıldı. Hayvanların bilgisayarlı tomografi (BT) görüntüleri alındıktan sonra, görüntüler MIMICS 20.1 programı ile yeniden yapılandırarak, iki boyutlu görüntülerden pelvik boşluğun üç boyutlu modeli elde edildi. Ortaya çıkan 3 boyutlu model üzerinde çap ve açı ölçümleri yapılarak morfometrik veriler elde edildi. Daha sonra tavşanlar diseke edilerek os coxae ortaya çıkarıldı ve anatomik oluşumlar isimlendirildi. Dişi ve erkek tavşanlarda pelvimetrik ölçümler karşılaştırıldığında dişilerde pelvik eğim dışındaki tüm değerlerin daha yüksek olduğu görüldü. Veriler, erkek ve dişi tavşanlar arasında sağ ve sol os coxae'nin hacmi ve yüzey alanı açısından anlamlı bir fark olmadığını ortaya koymaktadır ($p>0.05$). Dişi ve erkek Yeni Zelanda tavşanlarında pelvisin morfometrik farklılıklarının karşılaştırıldığı bu çalışmada hacim ve yüzey alanı verileri ilk kez paylaşıldı. Toplanan verilerin, tavşanlarda cinsiyet ayrımında kullanılabileceği, hekimlere hastalıkların teşhisinde yardımcı olacağı, klinik uygulamalara referans teşkil edeceği ve yeni araştırmalara temel oluşturacağı düşünülmektedir.

Anahtar Kelimeler: 3D modelleme, bilgisayarlı tomografi, Yeni Zelanda tavşanı, pelvik boşluk, pelvik kemikler

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Corresponding author/Sorumlu Yazar:
Muhammet Lütfi SELÇUK
E-mail: mlselcuk@kmu.edu.tr

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INTRODUCTION

The pelvis is the lower part of the trunk located between the abdomen and the hind limbs. The pelvis is formed by the articulation of the two os coxae at the ventral midline with the pubic symphysis and the os sacrum dorsomedially through the iliosacral joints. This frame connects the axial skeleton to the femurs and transfers the weight of the body to the hind limb.[1,2]

Animal dissections teach the structure of organs, function, and lay the foundation for advanced skills in sample preparation, comparative research, and veterinary practice. However, access to animal tissues limits the learning and study of anatomy through dissection, considering costs and ethical considerations.[3,4] In addition, the use of two-dimensional pictures in learning complex regions and the difficulty in imagining the three-dimensional structure make anatomy education difficult.[4,5] In recent years, three-dimensional models have begun to be preferred in anatomy education because they are practical to use and have a long duration of use. The development of three-dimensional scanning technology and the examination of tissues together with cross-sectional imaging methods increase the importance of three-dimensional anatomical models.[6,7] Computed tomography, one of the methods used in 3D modeling, is an imaging method that uses X-rays to create detailed pictures or scans of structures inside the body. CT scans images of bones, soft tissues, organs, and vessels within the body from different angles and allows them to be viewed in sections.[8] A 3D model of the desired structure is created from these images. In this way, the structures of organs can be observed, measurements can be made, animal research models can be developed, and it helps the physician in the diagnosis of bone diseases and surgical operations.[7,9]

The rabbit is a preferred experimental animal due to its high fertility, short generation period, and low cost. It is also used in the recognition of diseases in humans and animals as an excellent experimental clinico-anatomical example.[10,11] The pelvic cavity in rabbits, as in many mammals, plays a crucial role in supporting reproductive and digestive functions. The pelvic cavity in rabbits is formed by a set of fused bones, including os ilium, os ischii, and os pubis.[2,12] These bones articulate to create a sturdy pelvic girdle, providing support for the organs within. The pelvic bones' arrangement varies among species, influencing the overall shape and size of the pelvic cavity.[13,14] Rabbits are known for their prolific reproductive capabilities, making the understanding of their pelvic anatomy crucial.[10] Although there are many scientific studies that contribute to our understanding of the 3D anatomy of the pelvic cavity in humans and animals, no literature has been found except for a few studies[5,10] studying the anatomy of the pelvic cavity in rabbits. This study aims to obtain three-dimensional models of the cavum pelvis in New Zealand Rabbits of both genders using CT images, to measure the pelvis diameter and angles through the created digital models, and to compare female and male New Zealand rabbits in terms of sexual dimorphism.

MATERIALS AND METHODS

Materials

In the study, 20 healthy (10 females, 10 males) New Zealand rabbits (14 month old and weight of 2200-3500 g) cadavers were used. This study was approved by the Ethics Committee of Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center (Date: 02.11.2023; 2023-11/119).

Methods

New Zealand rabbits were placed individually on the CT device (Siemens, Somatom Sensation 64, Erlangen, Germany) in a prone position and symmetrically. CT device parameters were: physical detector collimation(32 x 0.5 mm), final section collimation (64 x 0.5mm), section thickness (0.50 mm), portal rotation time (330 msec), kVp (130), mA (300), resolution (512 x 512 pixels), resolution range (0.92 x 0.92).7,15 The images that were obtained were saved onto the hard disk in DICOM format. CT images were transferred to the MIMICS 21.0 (The Materialize Group, Leuven, Belgium) software program and threshold HU (Hansfield Unit) values were 350-1000 to distinguish bone tissues from other tissues. Then, separate 3D models of the bones forming the cavum pelvis (ossa coxae and os sacrum) were created using different tools of the program (region growing, edit mask, 3D calculation).

Morphometric Measurements

In this study, morphometric measurements were performed on the pelvic cavity and os coxae, 3D models of which were created. Volume and surface areas, diameter, and angle values were obtained from these models (Figure 1-3). Measurement points of the pelvis specified in the relevant literature were used.[5,14] The measurement points and their descriptions were given in Table 1.

Dissection Process

After CT imaging, the dissection of the pelvis was performed in New Zealand rabbits. First, using a surgical knife, the skin and muscles were carefully removed, leaving minimal soft tissue attachment to the bones. Then, the caudal border of

the last lumbar vertebra and the cranial border of vertebrae caudales and the sacrum were disconnected, and the caput ossis femoris was separated from the acetabulum and the dissection of the pelvic bones was completed. After these procedures, the pelvic bone was photographed (Nikon D5200, 18-55 Vr), and the anatomical structures were named (Figure 4). Nomina Anatomica Veterinaria (NAV 2017)[16] was used to name anatomical structures.

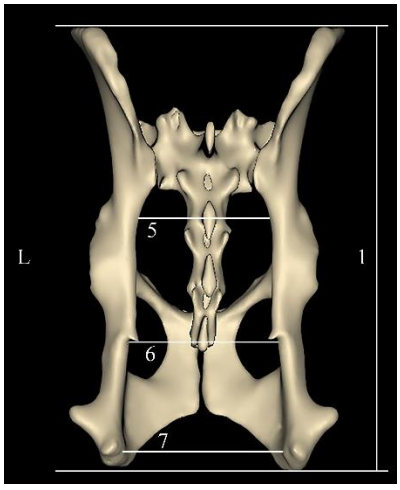

Figure 1. Measurements on facies dorsalis of pelvis.

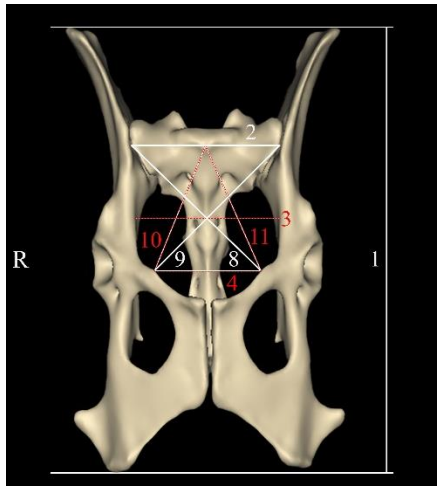

Figure 2. Measurements on facies ventralis of pelvis.

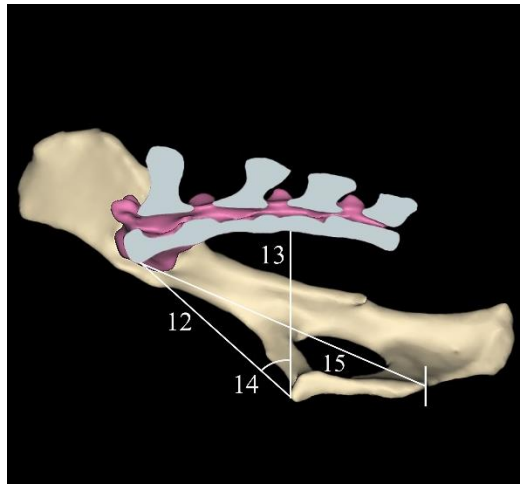

Figure 3. Measurements on lateral view of pelvis.

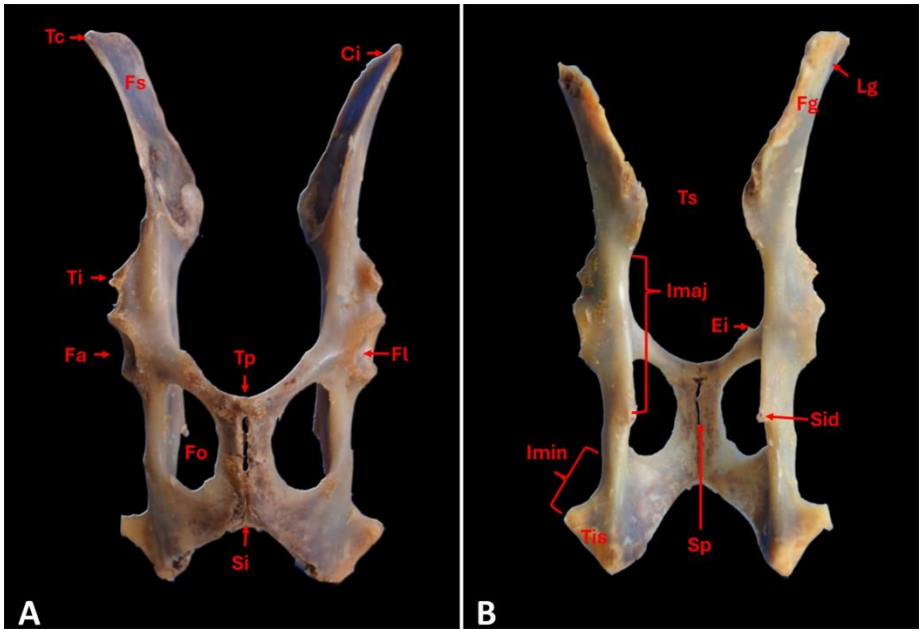

Figure 4. Os coxae in the New Zealand rabbit. **A: Ventral view**, Ti: Tuberositas iliaca, Fa: Fossa acetabuli, Fl: Facies lunata, Tc: Tuber coxae, Ci: Crista iliaca, Fo: Foramen obturatum, Fs: Facies sacropelvina, Tp: Tuberculum pubicum, Si: Symphysis ischiadica, **B: Dorsal view**, Fg: Facies glutea, Lg: Lineae gluteae, Ts: Tuber sacrale, Ei: Eminentia iliopubica, Sid: Spina ischiadica dorsale, Tis: Tuber ischiadicum, Imaj: Incisura ischiadica major, Imin: Incisura ischiadica minor, Sp: Symphysis pelvina

Table 1. Osteometric parameters and measuring points.[5,14]

Parameter	Abbreviation	Osteometric Parameters	Measuring Points
1	GL	Greatest length	Cranial border of ilia (margo iliocranialis)-most caudal points of the ischia
2	DTD	Dorsal transverse diameter	The distance between ends of two ala ossis sacri
3	ITD	Intermediary transverse diameter	The distance between the two tuberculum m.psoas minor.
4	VTD	Ventral transverse diameter	The distance between the two iliopubic eminence.
5	CrTD	Cranial transverse diameter	The distance between the front ends of two incisura ischiadica major
6	MTD	Medial (bispinous) transverse diameter	The distance between two spina ischiadica
7	CaTD	Caudal (bituberous) transverse diameter	The distance between the interior faces of two tuber ischiadicum (diameter between ischial tuberosities)
8	ROD	Right oblique diameter	The distance between the right sacroiliac joint and the left iliopectineal eminence
9	LOD	Left oblique diameter	The distance between the left sacroiliac joint and the right iliopectineal eminence
10	RSD	Right sacrocotyloid diameter	The distance between the promontory of the sacrum and the right iliopectineal eminence
11	LSD	Left sacrocotyloid diameter	The distance between the promontory of the sacrum and the left iliopectineal eminence
12	CV	Conjugata vera	The distance between the cranial end of the pelvic symphysis and the promontory of the sacrum
13	VD	Vertical diameter	The distance between the cranial end of pelvic symphysis and the ventral surface of the sacrum
14	PI	Pelvic inclination	The angle between the conjugate and vertical diameters
15	CD	Conjugate diagonalis	The distance between the caudal end of the pelvic symphysis and the promontory of the sacrum

Statistical Analysis

In the study, the analysis of morphometric data obtained from bones was performed using the SPSS 21.0 (IBM SPSS Corp., Armonk, NY, USA) statistical package program. The conformity of the variables to normal distribution (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests) were examined. As a result, the data shows a normal distribution. Paired samples t test was used for statistical comparisons of right and left values of bones, and Independent Samples t test was used for comparisons of male and female rabbits. The relationship between measurements was determined using Pearson's correlation analysis.7 Data are expressed as means ± standard deviation (SD). $P < .05$ was accepted statistically significant.

RESULTS

In the rabbit, the pelvic cavity was formed by the os coxae, which connects with symphysis pubica in the ventral midline, and the sacrum, which articulates dorsomedially through the iliosacral joints and first caudal vertebrae. Os coxae was formed by the union of three bones, os ilium located in the most cranial part, os ischii located in the dorso-caudal part and os pubis located in the ventro-caudal part. The acetabulum was located at the junction of these three bones. The articular surface (facies lunata) was crescent-shaped and the ends of this crescent were extending and turning into a foramen. The two bones were joined in the ventral midline by the symphysis pubica. The gluteal surface of the os ilium was divided by linea gluteae, which was a thick line. On the side facing the pelvic cavity, there was also a facies auricularis that would joint with the sacrum. There was a sharp crista iliaca on the cranio dorsal edge of the os ilium. Os ischii formed the dorso-caudal part of os coxae. The dorsal wide edge of the bone extended towards the acetabulum and raised towards the tuber ischiadicum. In addition, the os ischii was also shaping the spina ischiadica at the level of the acetabulum and the incisura ischiadica minor in an inverted v shape. Eminentia iliopubica was visible towards the medial part of the pecten ossis pubis.

Morphometric measurements made on the 3D model of the pelvis in New Zealand rabbits are given in Table 2. When pelvimetric measurements were compared in female and male rabbits, all values except pelvic inclination were found

to be greater in females. It was determined that there was a statistical difference between female and male rabbits in greatest length, dorsal transverse diameter, right and left oblique diameter, right and left sacrocotyloid diameter, and conjugata vera at $P < .05$ and there was a statistical difference in intermediary, ventral, cranial, transverse, caudal and vertical diameters at $P < .001$. But pelvic inclination did not show a statistical difference ($P > .05$). When comparing the right and left oblique diameter and sacrocotyloid diameter values of female and male rabbits, no statistical difference was found ($P > .05$).

Table 2. Morphometric measurement values of the pelvic cavity obtained from 3D reconstruction

Measurement	Gender	Mean	SD	Minimum	Maximum	*P*
Greatest length (mm)	Female	86.32	2.94	81.95	91.65	.031*
	Male	83.35	2.72	78.25	86.34	
Dorsal transverse diameter (mm)	Female	28.23	1.85	24.40	30.99	.002*
	Male	25.56	1.53	22.39	28.03	
Intermediary transverse diameter (mm)	Female	27.86	1.11	26.57	29.55	.000**
	Male	23.73	0.91	22.16	25.49	
Ventral transverse diameter (mm)	Female	21.83	1.50	20.19	24.38	.000**
	Male	17.34	1.08	16.01	20.02	
Cranial transverse diameter (mm)	Female	25.85	0.85	24.99	27.12	.000**
	Male	22.27	0.94	20.79	23.76	
Medial (bispinous) transverse diameter (mm)	Female	23.92	0.98	22.53	25.23	.000**
	Male	19.90	1.07	18.33	22.03	
Caudal (bituberous) transverse diameter (mm)	Female	29.70	2.31	25.66	33.32	.000**
	Male	23.01	1.62	19.61	24.84	
Right oblique diameter (mm)	Female	35.26	1.84	32.89	38.30	.012*
	Male	32.95	1.86	29.07	35.22	
Left oblique diameter (mm)	Female	36.17	1.86	33.07	39.28	.003*
	Male	33.44	1.65	29.83	35.44	
Right sacrocotyloid diameter (mm)	Female	27.68	1.26	25.64	29.84	.028*
	Male	26.31	1.31	24.03	28.16	
Left sacrocotyloid diameter (mm)	Female	27.46	1.06	25.99	29.15	.044*
	Male	26.21	1.61	23.02	28.46	
Conjugata vera (mm)	Female	33.10	1.57	29.97	35.66	.003*
	Male	30.74	1.56	28.30	33.53	
Vertical diameter (mm)	Female	25.61	1.28	23.15	27.35	.000**
	Male	22.87	1.02	21.69	24.79	
Pelvic inclination (°)	Female	55.37	6.19	45.52	64.99	.186
	Male	58.75	4.68	53.38	68.63	
Conjugate diagonalis (mm)	Female	52.12	2.10	48.80	55.94	.040*
	Male	49.36	1.60	47.23	51.97	

*$P < .05$, $P < .001$; independent samples t test

Table 3 presents the volume and surface area of os coxae obtained from 3D reconstruction. The data reveals that there is no significant difference in the volume and surface area of right and left os coxae between male and female rabbits ($P > .05$).

When the correlation of data obtained from female and male rabbits is examined in Table 4, there is a statistically significant correlation between all data except MTD, CaTD, and PI values in male rabbits, and CaTD in female rabbits.

Table 3. Os coxae volume and surface area obtained from 3D reconstruction

Gender	Measurement	Mean	SD	Minimum	Maximum	*P**
Female	Left os coxae volume (mm^3)	4253,43	275,60	3792,27	4594,42	.741
	Right os coxae volume (mm^3)	4246,52	299,38	3825,9	4718,08	
	Left os coxae surface area (mm^2)	4701,24	351,49	4047,47	5026,17	.059
	Right os coxae surface area (mm^2)	4671,89	362,03	4033,57	5036,49	
Male	Left os coxae volume (mm^3)	3796,62	223,76	3325,61	4019,64	.709
	Right os coxae volume (mm^3)	3791,51	206,45	3356,16	3989,44	
	Left os coxae surface area (mm^2)	4364,63	358,55	3805,35	4844,88	.424
	Right os coxae surface area (mm^2)	4378,23	350,52	3828,71	4788,24	

*$P < .05$; paired samples t test

Table 4. Correlation analyses of the pelvic cavity

	GL	DTD	IDT	VTD	CrTD	MTD	CaTD	ROD	LOD	RSD	LSD	CV	VD	PI	CD
GL	1	0.810**	0.474	0.208	0.338	-0.147	0.327	0.876**	0.888**	0.765*	0.725*	0.787**	0.558	0.128	0.602
DTD	0.757*	1	0.611	0.405	0.676*	0.055	0.528	0.738*	0.949**	0.570	0.594	0.690*	0.360	0.469	0.588
IDT	0.539	0.639*	1	0.859**	0.856**	0.457	0.544	0.186	0.600	0.042	0.123	0.166	0.375	0.279	0.296
VTD	0.362	0.501	0.715*	1	0.659*	0.240	0.444	0.008	0.431	-0.157	-0.110	-0.064	0.280	0.441	0.180
CrTD	0.463	0.463	0.803**	0.253	1	0.612	0.603	0.130	0.551	-0.011	0.115	0.175	0.061	0.459	0.174
MTD	0.257	0.160	0.644*	0.270	0.661*	1	0.161	-0.333	-0.095	-0.266	-0.127	-0.063	0.106	-0.171	-0.097
CaTD	-0.027	0.178	0.576	0.547	0.341	0.630	1	0.325	0.531	0.173	0.261	0.097	0.229	0.441	0.151
ROD	0.724*	0.788**	0.791**	0.752*	0.485	0.488	0.308	1	0.866**	0.943**	0.922**	0.868**	0.568	0.089	0.780**
LOD	0.669*	0.902**	0.738*	0.605	0.482	0.350	0.253	0.929**	1	0.723*	0.735*	0.760*	0.521	0.356	0.721*
RSD	0.372	0.690*	0.741*	0.641*	0.550	0.400	0.267	0.846**	0.880**	1	0.972**	0.927**	0.626	-0.202	0.784**
LSD	0.676*	0.757*	0.918**	0.714*	0.702*	0.664*	0.478	0.950**	0.891**	0.843**	1	0.887**	0.618	-0.179	0.844**
CV	0.148	0.455	0.459	0.785**	-0.048	0.181	0.362	0.730*	0.685*	0.729*	0.607	1	0.586	-0.150	0.714*
VD	0.064	0.168	0.296	0.548	-0.087	0.445	0.344	0.597	0.448	0.505	0.505	0.831**	1	-0.398	0.689*
PI	0.214	0.099	-0.222	-0.573	0.026	-0.041	-0.055	-0.319	-0.158	-0.476	-0.249	-0.646*	-0.621	1	-0.115
CD	0.382	0.775**	0.385	0.272	0.327	-0.214	-0.195	0.514	0.734*	0.708*	0.460	0.393	0.006	-0.147	1

*$P < .05$. ** $P < .01$; Gray cells in the table are data for male New Zealand rabbits.

DISCUSSION

The pelvis is crucial for stabilizing the spine and transferring movement through the sacrum to the rest of the body. The bones that make up the pelvis protect the organs located in this space and play an important role in birth. Pelvic dimensions play a crucial role in birth and reproductive processes.12,17 Moreover, pelvic bones can be used as an important parameter for gender determination.18 It is crucial to create three-dimensional reconstructions of bones because it allows surgeons to plan operations more accurately and examine the models from any desired angle.6 The reproducibility of the modeling method used, the ability to produce scientifically proven accurate results, and the ability to make measurements are among the reasons that increase its clinical importance.7,19 In this study, three-dimensional modeling of the pelvis was performed on CT images of New Zealand rabbits of the same age and different genders. Diameter and angle measurements were made on this model and the anatomical formations of the bones forming the pelvis were named by dissection.

El-Ghazali and El-Behery10 state that the os coxae in the rabbit consist of three bones that meet in the acetabulum and that the articulation surface with the femur is crescent-shaped. They also state that the wing of the os ilium is paddle-like, that it has a C-shaped auricular surface (Facies auricularis) for articulation with the wing of the sacrum on the surface facing the pelvis, that the crista iliaca is thin, and that this thinness continues the lateral and medial edges. Some literature states that there is an accessory bone called os acetabuli in the rabbit, which helps to form the acetabulum together with os ilium and os ischii.20,21 While the presented study is compatible with the data of El-Ghazali and El-Behery, os acetabuli was not found. It was determined that the acetabulum had a crescent-shaped articular surface and the end parts of the articular surface expanded and turned into a fovea.

In their comparative study on female and male New Zealand rabbits, Özkadif et al.[5] found a statistical difference between dorsal transverse, cranial transverse, caudal transverse, medial transverse, right oblique and left oblique diameters, conjugate vera, conjugate diagonalis, vertical diameter and inclinatio pelvis. They stated that there was no difference in terms of intermediate transverse, ventral transverse, right sacrocotyloid and left sacrocotyloid diameter values. In the presented study, while only pelvic inclination showed no difference, a statistical difference was detected in other diameter measurements. It was suggested that the difference in results between the two studies might have been due to the dissimilar ages of the rabbits. In a study conducted by Özkadif et al.[5], it was found that there was no significant difference between the right and left oblique diameter as well as the sacrocotyloid diameter values in female and male rabbits. Similar results were obtained in the presented study.

According to the literature, there was a greater distance between the symmetrical parts of the pelvis and a larger angle between the arcus ischiadicus in female animals.2 The presented study is compatible with the literature knowledge. It is also observed that the measured diameter values were larger in female rabbits compared to males. It is believed that the larger size of the female pelvic cavity is due to the presence of organs of the urinary system and the birth canal required for reproduction.

In osteometric diameter measurements of the pelvis using radiological imaging on cats18,22, Kangal dogs23, gazelle14 and red fox12, it was stated that the values in males were higher than in females. In New Zealand rabbits, females had higher values in all pelvic diameter measurements except the intermediary transverse diameter.5 The presented study concluded that pelvic diameters were higher in female rabbits than in males, excluding pelvic inclination.

In conclusion, pelvis data obtained from 3D reconstructions using CT images in healthy New Zealand rabbits revealed morphometric differences of the pelvic cavity in females and males, and volume and surface area data were shared for the first time. The markings on the pictures are expected to aid researchers in comprehending the anatomical structure of the area. The acquired data could be used in rabbit sex discrimination, would assist physicians in diagnosing patients in the clinic, serve as a reference for clinical practices, and establish the foundation for new research.

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Evaluation of Economic Efficiencies of Veterinary Practices in Kars Province with Data Envelopment Analysis

Kars İlindeki Veteriner Hekim Muayenehanelerinin Ekonomik Etkinliklerinin Veri Zarflama Analizi ile İncelenmesi

Erol AYDIN[1]
Can İsmail ZAMAN[1]
Mehmet KÜÇÜKOFLAZ[1]
Merve AYYILDIZ AKIN[2]

[1]Kafkas University, Faculty of Veterinary Medicine, Services, Department of Animal Health Economics and Management, Kars, Türkiye
[2]Kafkas University, Faculty of Veterinary Medicine, Services, Department of Biostatistics, Kars, Türkiye

ABSTRACT

This study was performed to determine the economic activity and profitability of veterinary practices operating in Kars province by using Data Envelopment Analysis (DEA). In this study, the 2023 data of 53.6% of veterinary practices operating in Kars province were obtained through a face-to-face survey. In the DEA applied to determine the economic efficiency of practices, input-oriented constant returns to scale (CCR) technique were used According to the study findings, 6.7% of the veterinarians participating in the survey were female, 93.3% were male, and 46.6% were aged between 25 and 29 years. It was observed that all the veterinarians participating in the research had been practicing their professional activities since the date of graduation, and the majority (73.3%) had 3-12 years of experience (graduate date 2011-2020). It was determined that 10% of the veterinarians participating in the survey received postgraduate education (master's degree with thesis and PhD). It was found that 30% of veterinarians opened a practice after working in a practice. According to the DEA results for economic efficiency, it was determined that 90% of the practices were active. As a result, when veterinary practices were evaluated in terms of efficiency, it was understood that rent, maintenance-repair, general administrative costs, and other costs must be reduced in order to make economically ineffective veterinary practices to effective.

Keywords: Kars, practice, economic efficiency, data envelopment, veterinarian

ÖZ

Bu çalışma ile Kars ilinde faaliyet gösteren veteriner hekim muayenehanelerinin ekonomik aktiflik ve karlılık durumlarının Veri Zarflama Analizi (VZA) kullanılarak belirlenmesi amaçlanmıştır. Çalışmada, Kars ilinde faaliyet gösteren veteriner hekim muayenehanelerinin %53,6'sının 2023 yılına ait verileri yüz yüze uygulanan anket ile elde edilmiştir. Muayenehanelerin ekonomik açıdan etkinliklerinin tespiti için uygulanan VZA'da girdi yönlü ölçeğe göre sabit getiri tekniği (CCR) kullanılmıştır. Çalışma bulgularına göre, ankete katılan veteriner hekimlerin %6,7'si kadın, %93.3'ü ise erkek olup, %46,6'sının 25-29 yaş aralığında olduğu tespit edilmiştir. Araştırmaya katılan veteriner hekimlerin mezuniyet tarihinden itibaren tamamının meslek faaliyetlerini icra ettiği görülmüş ve çoğunluğunun (%73,3) 3-12 yıllık (mezuniyet tarihi 2011-2020) tecrübeye sahip olduğu saptanmıştır. Ankete katılan veteriner hekimlerin %10'unun lisansüstü eğitimi (tezli yüksek lisans ve doktora) aldığı tespit edilmiştir. Veteriner hekimlerin %30'unun daha önce bir muayenehanede çalıştıktan sonra muayenehane açtığı görülmüştür. Ekonomik etkinlik için yapılan VZA sonuçlarına göre muayenehanelerin %90'nın aktif olduğu tespit edilmiştir. Sonuç olarak işletmeler etkinlik yönünden değerlendirildiğinde, ekonomik yönden etkin olmayan işletmelerin etkin olabilmesi için kira, bakım-onarım, genel idare giderleri ve diğer giderlerin azaltması gerektiği anlaşılmaktadır.

Anahtar Kelimeler: Kars, muayenehane, ekonomik etkinlik, veri zarflama, veteriner hekim

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Corresponding author/Sorumlu Yazar:
Erol AYDIN
E-mail: dr-erolaydin@hotmail.com

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INTRODUCTION

Veterinary medicine, all around the world, is considered one of the deep-rooted professions with an important past.[1] The foundation of the veterinary profession began with the opening of a school providing veterinary education in France in 1762 and has continued until today.[2] In Türkiye, academic veterinary education began in 1842, 80 years after the first veterinary school established in the world, by a Prussian military veterinarian named Godlewsky, by giving veterinary medicine courses to cavalry schools in Istanbul.[3,4] Today, there are 32 veterinary faculties operating academically in Türkiye.[5] Individuals who receive a 5-year undergraduate education from these faculties and are successful receive the title of veterinarian. Veterinarians, who graduate from veterinary faculties, are employed in many different fields and units in the public and private sectors. Veterinarians can work in ministries, municipalities, and universities in the public sector. In addition, they can work as clinicians in the field of horses, cattle-sheep, poultry, and animals in the private sector as well as in veterinary practices operating in these fields and the pharmaceutical production and marketing sector and others.[6] Veterinarians work in different sectors such as improving animal production (increasing productivity, breeding animals, protecting genetic resources, etc.), preventing and treating animal diseases, protecting public health (zoonotic diseases, etc.), and providing food services at all stages from production to consumption of animals and animal products as a professional group responsible and authorized for safety.[7,8] It is extremely important for sustainability that veterinarians, especially those who own private practices, undertake such important tasks and can run an economically profitable veterinary practice. A profitable veterinary practice is possible if veterinarians fulfill their entrepreneurship, management, and physician missions in the best possible way. Many studies reported that practices managed by veterinarians trained in veterinary practice management and administration were better in terms of both veterinary service quality and profitability.[9,10] It is regarded as necessary to analyze the economic situation of veterinary practices at certain time intervals to evaluate service quality and profitability. Data envelopment analysis is used in the economic evaluation of practices, as in the current study.

Data envelopment analysis, which dates back to Farrell's work in 1957, was first developed by Charnes, Cooper and Rhodes[11] as a method to evaluate the comparative efficiency of organizational units.[12-15] Data envelopment analysis is a non-parametric linear programming method that uses multiple inputs to obtain multiple outputs. In this respect, it differs from other single-output parametric methods. In DEA, efficiency scores vary between 0 and 1. It is suggested that the effectiveness of the decision-making units (DMU) whose value is closest to 1 is more effective than the others. Veterinary practices with an efficiency score of 1 are considered fully efficient.[16] In the literature review, no study evaluating the economic efficiency of veterinary practices using DEA was found.

The objective of this study was to evaluate the economic efficiency of veterinary practices operating in Kars province with DEA by considering income and cost variables.

MATERIALS AND METHODS

This study was approved by the Kafkas University Non-Interventional Clinical Research Ethics Committee (Approval date and number: 29.09.2023 and 8/123).

Study Design

In this study, data obtained from 30 veterinary practices out of a total of 56 veterinary practices operating in all districts (8 districts) of Kars province, who agreed to participate in the survey, were used. One-year data on veterinary practices were obtained from face-to-face surveys (general information about the practice and the veterinarian who owns the practice, information about the costs and income of the practice). The study results were examined in the following three parts.

Descriptive information about veterinary practices: Survey questions were asked to veterinarians who own practice, including general information about them and their practices. At the end of the survey, the answers given to the questions were presented as percentages.

Cost, income and profitability of practices:
Survey questions were asked to veterinarians to obtain 10 input and five output data for their practices.

The economic analysis method for determining total cost, income and profit situations was presented below.

Total cost (TRY) = invoice (communication, electricity, water) cost + medical equipment cost + maintenance-repair (practice, vehicle) cost + medicine and food supplement cost + rent cost + employee cost + artificial insemination (sperm, straws, etc.) cost + accessory (collar, etc.) cost + depreciation cost + general administrative cost (transportation + communication + health insurance) cost

Total income (TRY) = Examination fee (veterinarian + travel fee) + medicine and food supplement (food, premix) fee + artificial insemination fee + accessory fee (leash, etc.) + additional income (expertise, consultancy, etc.)

Profit (TRY) = total income - total cost

Statistical Analysis:

In the created DEA economic efficiency model, invoice (communication, electricity, water), medical equipment, maintenance-repair (practice, vehicle), medicine and food supplements, rent, employee, artificial insemination (semen, straws, etc.), accessories (collar, etc.), depreciation, general administration (transportation + communication + health insurance) costs were taken into account as 10 input variables of veterinary practices.

Economic efficiency scores were calculated by DEA analysis (Table 1).

Table 1. Income and cost items

Costs Items	Income Items
1. Invoice (communication, electricity, water)	1. Examination fee (veterinarian + travel fee)
2. Medical equipment	2. Medicine and food supplement (food, premix) fee
3. Maintenance-repair (practice, vehicle)	3. Artificial insemination fee
4. Medicine and food supplement	4. Accessory fee (leash, etc.)
5. Rent	5. Additional income (expertise, consultancy, etc.)
6. Employee	
7. Artificial insemination (sperm, straws, etc.)	
8. Accessory (collar, etc.)	
9. Depreciation	
10. General administrative (transportation + communication + health insurance)	

The obtained data were analyzed by applying the input-oriented CCR technique. In DEA, it was assumed that each unit on the problem to be analyzed has "m" inputs, "s" outputs and "n" decision-making units. The ith input amount of the jth decision-making unit was Xij ≥ 0 and the Yij parameter indicated the ith output amount used by the jth decision-making unit. The mathematical expression of the CCR technique of the input-oriented fractional DEA model, where Yij ≥ 0, was as follows:

$$Enb\ h_k = \frac{\sum_{r=1}^{s} u_{rk} y_{rk}}{\sum_{i=1}^{m} v_{ik} X_{ik}} \quad [1.1]$$

$$= \frac{\sum_{r=1}^{s} u_{rk} y_{rj}}{\sum_{i=1}^{m} v_{ik} X_{ij}} \leq 1 \quad ; j = 1,2,\dots,n \quad [1.2]$$

$$u_{rk} \geq 0; \quad r = 1,2,\dots,s\ ,\ v_{ik} \geq 0; \quad i = 1,2,\dots,m \quad [1.3]$$

CCR (constant returns to scale) data envelopment model can be obtained by converting the above model to the following model.

$$Enb\ h_k = \sum_{k=1}^{s} u_{rk} y_{rk}\ ;\ \text{k=1,2,...,n} \quad [2.1]$$

$$\sum_{i=1}^{m} v_{ik} X_{ik}\ , \quad u_{rk} \geq 0; \quad r = 1,2,\dots,s \quad v_{ik} \geq 0; \quad i = 1,2,\dots,m \quad [3.1]$$

$$\sum_{r=1}^{s} u_{rk} y_{rj} - \sum_{i=1}^{m} v_{ik} X_{ij} \leq 0 \ ;\ \text{j = 1, 2,...,n} \quad [4.1]$$

The model is represented as follows. In the model;
Enb : Maximization
urk": Weight assigned to the rth output by decision unit k,
vik": Weight assigned to the ith input by decision unit k,
Yrk": Output produced by decision unit k for the rth output,
Xik": ith input used by decision unit k,
Yrj": rth output produced by jth DMU,
Xij": ith input used by jth DMU,

The problem here is processed n times to determine the effectiveness of all DMU scores, and weighted inputs and outputs are selected to optimize the efficiency score of each decision-making unit. The efficiency value of each DMU is in the range of 0-1. If the efficiency value of the DMU is 1, the relevant decision-making unit is considered effective, and these also constitute the efficiency limit. If the efficiency value of the DMU is less than 1, the relevant decision-making unit is ineffective[12, 17].

The analysis was applied to a total of 30 veterinary practices. Inputs were coded for the 1st Practice as “I1 {I},” for the 2nd Practice as “I2 {I}” and for the 30th Practice as "I30 {I},” and the outputs were coded for the 1st Practice as "O1 {O},” for the 2nd Practice as "O2 {O}"and for the 30th Practice as "O30 {O}.” MS Excel[18] and EMS (Efficiency Measurement System) version 1.3.0[19] were used for DEA in the study, and IBM SPSS 25.0[20] package program was used for the independent sample t test.

Table 2. Some general information about veterinary practices

General Information About Practices	%
1. Gender of veterinarians	100
Male	93.3
Female	6.7
2. Age of the veterinary practice owner veterinarian	100
25-29	46.6
30-34	13.3
35-39	33.4
40 and above	6.7
3. University graduation year	100
2001-2010	16.6
2011-2020	73.3
2021-2023	10.1
4. Status of continuing postgraduate education after undergraduate graduation	100
Yes	10.0
No	90.0
5. Have you attended any training courses and seminars organized within the scope of professional education?	100
Yes	93.3
No	6.7
6. Have you ever worked at a veterinary practices before	100
Yes	30
No	70

RESULTS

Table 2 presents general information about veterinary practices.

In this study, 6.7% of the veterinarians participating in the survey were female, 93.3% were male, and 46.6% were aged between 25 and 29 years. It was found that all the veterinarians participating in this study had been practicing their professional activities since the date of graduation, and the majority (73.3%) had 3-12 years of experience (graduation date 2011-2020). It was determined that 10% of the veterinarians participating in the survey had postgraduate education (master's degree with thesis/PhD degree). It was determined that veterinarians were interested in seminars and courses, and almost all of them (93.3%) participated in such training activities. It is observed that 30% of veterinarians opened a practice after gaining experience in a practice.

Data regarding the establishment and operation period of veterinary practices are given in Table 3.

The majority of veterinarians (76.7%) answered no to the question "Have you ever had concerns about your competence when opening a practice?". The ideal of opening a practice (38.7%), desire to work independently (38.7%), 12.9% considering the opportunities to work in the public sector as limited, and willingness to earn a high income (9.7%) were among the reasons for opening a practice among the veterinarians surveyed in the present study. It was determined that 86.7% of the veterinarians participating in the survey met their financing needs from equity capital in the establishment of their practices, while 13.3% used loans. Knowing the environment (42.4%), knowing the animal population in the region (39.4%), the number of practices and their proximity to each other (9.1%), capital status (3.0%), and other factors (6.1%) were found to be effective in the selection of the location of the practice by veterinarians. Veterinarians stated that when determining the fee schedule in their practices, 73.3% considered their costs, 23.3% considered the fees of other practices, and 3.4% considered the minimum wage tariff determined by the chamber of veterinarians. When the veterinarians in charge of the practices were asked about the number of employees other than themselves; 15 of them (50%) said they had no other employee other than themselves, 6 of them (20%) worked with only 1 auxiliary staff, 5 of them (16.6%) employed 1 veterinary technician, 2 of them (6.7%) had 1 veterinarian and 1 assistant, and 2 of them (6.7%) stated that they worked with 2 veterinary technicians. It was seen that almost all the veterinarians in the current study examine and treat cattle, sheep, pets, poultry and exotic animals. It was determined that 96.7%

Table 3. Data regarding the establishment and operation period of veterinary practices

Data regarding the establishment and operation period of veterinary practices	%
1. Did you have any concerns about your competence when opening the practices	100
Yes	23.3
No	76.7
2. The reason for choosing the profession of clinical veterinarian	100
Professional idealism	38.7
Desire for independent work	38.7
Professional idealism Desire for independent work Limited opportunities for working in the public sector	12.9
High income potential	9.7
3. Did you use credit during establishment?	100
Yes	13.3
No	86.7
4. The factors influencing the choice of establishment location	100
Animal population in the region	39.4
Number of practices and their proximity	9.1
Familiarity with the environment	42.4
Capital situation	3.0
Other	6.1
5. What do you consider when determining fees for vaccination, examination, treatment, etc. at your practices?	100
By looking at the fees of other practices	23.3
Based on costs	73.3
By referring to the minimum fee schedule of the Veterinary Health Organization (VHO)	3.3
6. Number of employees	100
Only oneself	50.0
Himself + 1 Assistant Staff (secretary, etc.)	20.0
Himself + 1 Veterinarian + 1 Veterinary Technician	6.7
Himself + 1 Veterinary Technician	16.6
Himself + 2 Veterinary Technicians	6.7
7. What practices do you implement to increase the number of patients and ensure customer loyalty?	100
Establishing good relationships with animals owners	20.0
Improving service quality	70.0
Developing patient registration and tracking system	3.3
Providing services by visiting homes	6.7

of the patient profiles that came to these veterinarians' practices or where the veterinarian treated the patient on-site were cattle, 90% were sheep, 80% were pets, 80% were poultry and 23.3% were exotic animals (Table 3).

Problems encountered establishment and operation period of veterinary practices are given in Table 4.

Insufficiency of credit and financing opportunities (36.7%), lack/expensiveness of devices and equipment (30%), problems in promotion and public relations (20%) and bureaucratic procedures encountered during the establishment phase (10%) were among the problems veterinarians encountered when establishing a practice. It was determined that the most important problems experienced by veterinarians during the operation period were that the animal owner found the fee tariff high for the service and bargained with it, at a rate of 63.3%, and that the fees were reduced due to competition between practices, at a rate of 36.7%. In this study, it was observed that 73.3% of veterinarians could not collect the full fee they requested for the provided health services (Table 4).

Table 4. Problems encountered establishment and operation period of veterinary practices	
Problems encountered establishment and operation period of veterinary practices	%
1. The most significant challenge encountered when establishing a veterinary practice?	100
Competitive pressure among practices leading to downward pressure on fees	36.7
Animals owners finding service fees high and bargaining for lower rates	10.0
Low-income levels of animals owners	20.0
In some cases, abandonment of stray animals at the practices and failure to pay for treatment	30.0
Other	3.3
2. The most significant problem experienced during the operation period of the practices?	100
The reduction of fees due to competition among veterinary practices	36.7
Animals owners finding the service fees high	63.3
3. Are you able to collect the fee you request for the service provided in full?	100
Yes	26.7
No	73.3

Table 5. Annual average cost of veterinary practices

Cost Items	Number of Vet. practices	Average Costs (TRY)	Share of Total Costs (%)	$S_{\bar{x}}$
1. Invoice	30	24,292	1.8	2,455.3
2. Medical equipment	30	47,533	3.5	6,256.4
3. Maintenance-repair	30	35,140	2.6	2,698.5
4. Medicine and food supplement	30	856,400	62.4	107,265.7
5. Rent	30	40,740	3.0	4,283.1
6. Employee	30	104,400	7.6	24,694.4
7. Artificial insemination	30	29,680	2.2	6,146.7
8. Accessory	30	3,400	0.2	2,119.5
9. Depreciation	30	24,452	1.8	3,592.6
10. General administrative	30	206,282	15.0	19,301.3
Total	30	1,372,319	100	140,182.1

$S_{\bar{x}}$: Standart Error

The annual average cost items (TRY) of veterinary practices operating in Kars are given in Table 5 and Figure 1.

Proportional distribution of total annual costs of veterinary practices sorted from largest to smallest as medicine and food supplement costs (62.4%), general administrative costs (transportation + communication + social security) (15.0%), employee, ousts (7.6%), medical equipment costs (syringe, gloves, boots, overalls, apron) (3.5%), rent costs (3.0%), maintenance-repair costs (2.6%), artificial insemination cost (2.2%), depreciation costs (1.8%), invoice costs (electricity, natural gas, water) (1.8%) and accessory costs (collar, etc.) (0.2%) (Table 5; Figure 1).

Annual average incomes of veterinary practices are given Table 6.

When looking at the average annual income of veterinary practices, the highest income was received from the sale of medicines and food supplements (66.1%), followed by examination (veterinary and transportation) (27.3%), artificial insemination (4.8%), additional (expertise, consultancy, etc.) (1.5%), and accessory incomes (0.3%) (Table 6).

The economic efficiency scores of the practices are given in Table 7.

According to the DEA results, the efficiency score of 27 practices (Practices 1-9, 11-13, 15-30), which are among the decision-making units, was 1, whereas the efficiency score of the other three ineffective practices (Practices 7, 10, and 14) was 0.

Practice 9 (by Practice 10), Practice 11 (by Practice 7), Practice 19 (by Practice 14), Practice 20 (by Practice 10), Practice 23 (by Practice 14), Practice 27 (by Practice 7), Practice 28 (by Practice 14), were referenced once, and Practice 5 (by Practices 10 and 14), Practice 18 (by Practices 7 and 14) and Practice 25 (by Practices 7 and 14) were referenced twice (Table 7).

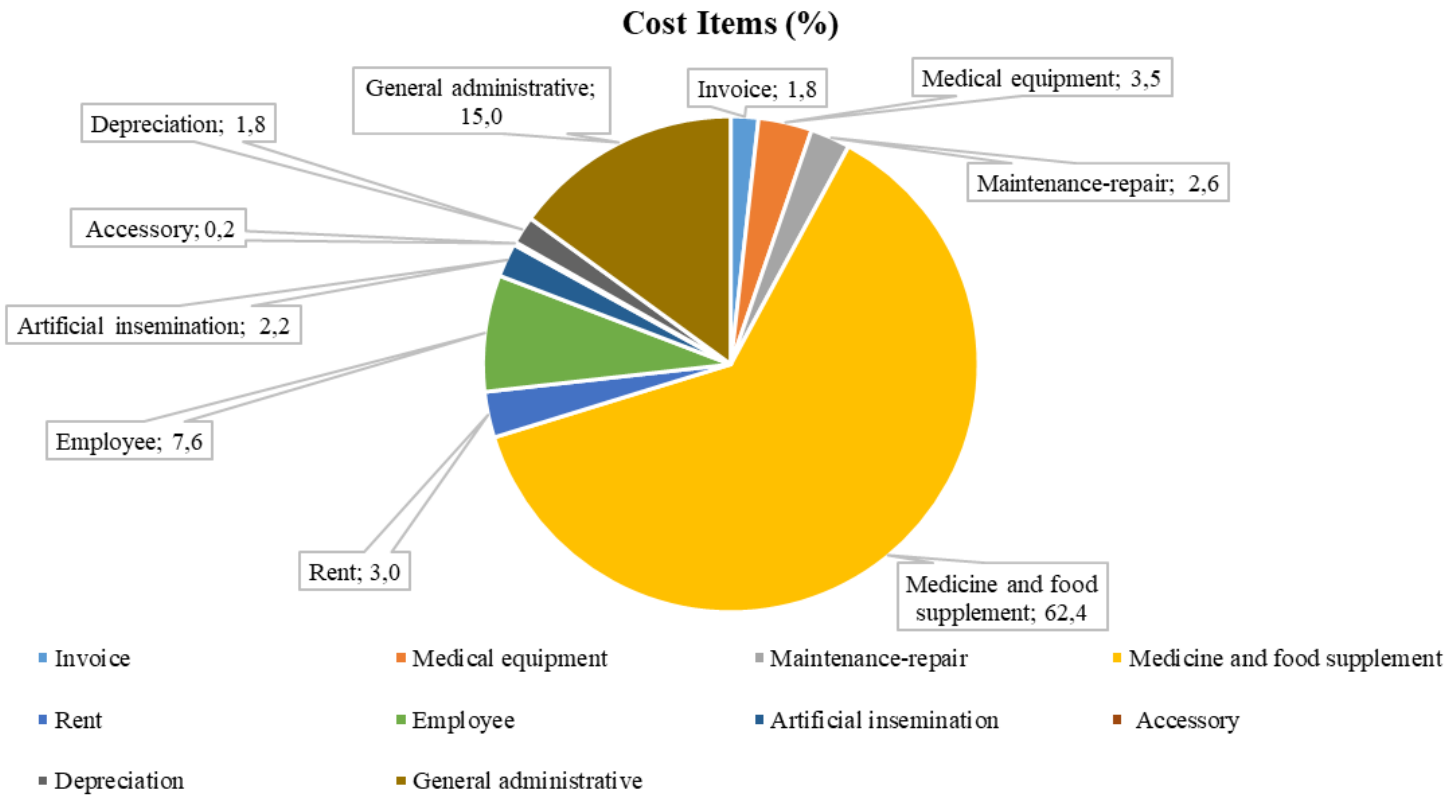

Figure 1. Proportional distribution of annual average cost items of veterinary practices (%)

Table 6. Annual average incomes of veterinary practices

İncome İtems	Number of Vet Practices	Average Annual Income	Share of Total Revenue (%)	$S_{\bar{x}}$
Examination	30	577,600	27.3	52,045.8
Medicine and food supplement	30	1,398,800	66.1	171,038.2
Artificial insemination	30	102,520	4.8	16,637.8
Accessory	30	31,200	1.5	14,799.1
Additional income	30	6,080	0.3	3,199.3
Total	30	2,108,920	100	204,668.9

$S_{\bar{x}}$: Standart Error

The CCR input efficiency score of the practices and the residual values of the variables (input elements that need to be reduced) are given in Table 8.

According to the input-oriented CCR efficiency analysis, considering the Practice 7, it was required that the annual invoicing cost by 1,624.5 TRY, the medical equipment cost by 4,774.3 TRY, the maintenance-repair cost by 3,006.6 TRY, the artificial insemination cost by 4,856.7 TRY, the depreciation cost by 8,688.8 TRY, and the general administrative costs by 16,189.2 TRY should be reduced in order to be effective.

In order to be effective, Practice 10 must reduce its annual invoice cost by 21,366.7 TRY, its medical equipment cost by 14,094.1 TRY, its maintenance-repair cost by 13,904.5 TRY, its rent cost by 9,732.7 TRY, its depreciation cost by 7,927.8 TRY and its general administrative costs by 68,080.8 TRY. It was determined that in order to be effective, Practice 14 should reduce its annual invoice costs by 23,312.3 TRY, its maintenance-repair costs by 15,500.2 TRY, its rent costs by 19,047.7 TRY, its employee costs by 53,696.8 TRY and its general administrative costs by 26,149.5 TRY (Table 8).

Table 7. Economic efficiency scores of veterinary practices

Decision unit (Vet Practices No)	Efficiency Score	Benchmarks (Reference set)	Number of References Shown by Another Decision Unit	Efficiency Status
Veterinary Practice 1	1	Veterinary Practice 1 (1.00)	0	Effective
Veterinary Practice 2	1	Veterinary Practice 2 (1.00)	0	Effective
Veterinary Practice 3	1	Veterinary Practice 3 (1.00)	0	Effective
Veterinary Practice 4	1	Veterinary Practice 4 (1.00)	0	Effective
Veterinary Practice 5	1	Veterinary Practice 5 (1.00)	2	Effective
Veterinary Practice 6	1	Veterinary Practice 6 (1.00)	0	Effective
Veterinary Practice 7	0.96	Veterinary Practice 11 (0.02); Veterinary Practice 18 (0.16); Veterinary Practice 25 (0.65); Veterinary Practice 27 (0.02)	0	Ineffective
Veterinary Practice 8	1	Veterinary Practice 8 (1.00)	0	Effective
Veterinary Practice 9	1	Veterinary Practice 9 (1.00)	1	Effective
Veterinary Practice 10	0.95	Veterinary Practice 5 (0.27); Veterinary Practice 9 (0.08); Veterinary Practice 20 (0.38)	0	Ineffective
Veterinary Practice 11	1	Veterinary Practice 11 (1.00)	1	Effective
Veterinary Practice 12	1	Veterinary Practice 12 (1.00)	0	Effective
Veterinary Practice 13	1	Veterinary Practice 13 (1.00)	0	Effective
Veterinary Practice 14	0.93	Veterinary Practice 5 (0.14); Veterinary Practice 18 (0.32); Veterinary Practice 19 (0.44); Veterinary Practice 23 (0.09); Veterinary Practice 25 (0.07); Veterinary Practice 28 (0.06)	0	Ineffective
Veterinary Practice 15	1	Veterinary Practice 15 (1.00)	0	Effective
Veterinary Practice 16	1	Veterinary Practice 16 (1.00)	0	Effective
Veterinary Practice 17	1	Veterinary Practice 17 (1.00)	0	Effective
Veterinary Practice 18	1	Veterinary Practice 18 (1.00)	2	Effective
Veterinary Practice 19	1	Veterinary Practice 19 (1.00)	1	Effective
Veterinary Practice 20	1	Veterinary Practice 20 (1.00)	1	Effective
Veterinary Practice 21	1	Veterinary Practice 21 (1.00)	0	Effective
Veterinary Practice 22	1	Veterinary Practice 22 (1.00)	0	Effective
Veterinary practice 23	1	Veterinary practice 23 (1.00)	1	Effective
Veterinary Practice 24	1	Veterinary Practice 24 (1.00)	0	Effective
Veterinary Practice 25	1	Veterinary Practice 25 (1.00)	2	Effective
Veterinary Practice 26	1	Veterinary Practice 26 (1.00)	0	Effective
Veterinary Practice 27	1	Veterinary Practice 27 (1.00)	1	Effective
Veterinary Practice 28	1	Veterinary Practice 28 (1.00)	1	Effective
Veterinary Practice 29	1	Veterinary Practice 29 (1.00)	0	Effective
Veterinary Practice 30	1	Veterinary Practice 30 (1.00)	0	Effective

Table 8. The input-oriented (CCR) efficiency score of veterinary practices and the residuals of variables

Vet. Practices	Eff. score	İnvoice	Med. equipment	Main. repair	Med. and food	Rent	Employee	Art. insemination	Accessory	Depreciation	Gen. administrative
1	1	0	0	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0	0	0
5	1	0	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0	0	0
7	0.6	1,624.5	4,774.3	3,006.6	0	0	0	4,856.7	0	8,688.8	16,189.2
8	1	0	0	0	0	0	0	0	0	0	0
9	1	0	0	0	0	0	0	0	0	0	0
10	0.95	21,366.7	14,049.1	13,904.5	0	9,732.7	0	0	0	7927,8	68080,8
11	1	0	0	0	0	0	0	0	0	0	0
12	1	0	0	0	0	0	0	0	0	0	0
13	1	0	0	0	0	0	0	0	0	0	0
14	0.93	23,312.3	0	1,500.2	0	19,047.7	53,696.8	0	0	0	26,149.5
15	1	0	0	0	0	0	0	0	0	0	0
16	1	0	0	0	0	0	0	0	0	0	0
17	1	0	0	0	0	0	0	0	0	0	0
18	1	0	0	0	0	0	0	0	0	0	0
19	1	0	0	0	0	0	0	0	0	0	0
20	1	0	0	0	0	0	0	0	0	0	0
21	1	0	0	0	0	0	0	0	0	0	0
22	1	0	0	0	0	0	0	0	0	0	0
23	1	0	0	0	0	0	0	0	0	0	0
24	1	0	0	0	0	0	0	0	0	0	0
25	1	0	0	0	0	0	0	0	0	0	0
26	1	0	0	0	0	0	0	0	0	0	0
27	1	0	0	0	0	0	0	0	0	0	0
28	1	0	0	0	0	0	0	0	0	0	0
29	1	0	0	0	0	0	0	0	0	0	0
30	1	0	0	0	0	0	0	0	0	0	0

Economic data for efficient and inefficient practices are given in Table 9.

Table 9. Average economic data for effective and ineffective veterinary practice

Variable	Group	
	Effective	Inefficient
Cost	1,409,253	1,039,920
Income	2,195,778	1,400,000
Profit	786,525	360,080

According to the results of the study, the average costs, income, and profitability of effective practices were higher. It was determined that economically efficient veterinary practices had an average of 426,445 TRY more profit than inefficient veterinary practices in terms of profitability (Table 9).

DISCUSSION

Although veterinary practices are established as veterinary practices that aim to provide treatment, care, and other services for animal health, they are essentially commercial veterinary practices. Veterinary practices, as a commercial enterprise, must have the necessary competencies in customer relations, service quality methods, patient registration systems, patient tracking, and analysis of inputs and outputs to be economically profitable / effective in terms of sustainability as well as the professional knowledge of veterinarians.[21]

In the present study, 53.6% (30 practices) of 56 practices operating in Kars were included. In other words, more than half of the total practices operating in Kars province were included in this study. In some previous studies, participation rates were reported as 20.8% [22] and as 47.1% [23]. It was determined that 6.6% of the veterinarians participating in this study were women and 93.4% were men, and almost all of them worked as cattle and sheep physicians. In some studies, although the rate of female veterinarians was higher than that in the present study, it was observed that the rate of female veterinarians, especially in veterinary practices focusing on pet animals, was higher than that of cattle.[23,24] There were studies in the literature reporting that the rate of female veterinarians was lower than the rate of male veterinarians as in the present study.[25-27] The high interest of female veterinarians in pet animals[28], the fact that female veterinarians do not consider working primarily in cattle as a priority, the majority of the studies conducted in cattle and ovine practices, as in the present study, the fact that high cattle populations in the region where the present study was conducted (Kars province ranks 4th in terms of the presence of cattle in Türkiye), and the conditions for practicing cattle and ovine medicine are more difficult than pet, exotic, and poultry medicine (many problems such as high power requirements of animal restraint, on-site intervention to the patient, time, and climate) may be among the reasons for this situation.[29] It was determined that the highest rate of age and experience of the veterinarians participating in the study was in the 25-29 age group with 46.6%, and the majority of them had 3-12 years of experience. There are studies with similar findings in terms of the age[27] and experience of veterinarians.[23,27] Additionally, when the opening years of the practices were evaluated, it was noteworthy that the number of practices opened, especially in the last 5 years was very high. The fact that experience of veterinarian was between 3-12 years, which was also mentioned in previous studies[19,20] might be explained by the increasing number of veterinary faculties in recent years and limited employment opportunities in the public sector. It was observed that 90% of the veterinarians in this study did not receive a master's degree with a thesis or PhD after their undergraduate education. The rate of veterinarians without postgraduate education found by Aral et al.[23] in Ankara and Erdoğan and Sarıözkan[27] in Nevşehir was consistent with the rate found in the present study. The low rate of veterinarians receiving postgraduate education can be attributed to their inability to spare time for education because of working overtime (24/7 working principle). However, according to the findings of both this study and other studies[23], the participation rate of physicians in courses and seminars with short/limited training periods was found to be high.

It has been observed that the examination service fee and fees of medicine and materials sale were mostly determined by veterinarians by looking at the sales prices, costs and fees of other practices. Similar findings were also found in the study conducted by Erdoğan and Sarıözkan.[27] In order to prevent such unfair competition and practices that may negatively affect patient owners, the necessary authorities need to inspect practices and take the necessary measures in terms of fee schedule application.

It was observed that 42.4% of veterinarians considered the environment when choosing a practice location, 39.4% considered the animal population in the region, 9.1% considered the number of practices and their proximity to each other, 3.0% considered the capital situation, and 6.1% considered other reasons. Previous studies also reported that veterinary practices considered the animal population and household income level in the region when choosing a location for establishment.[22,23] Possible reasons why veterinarians should pay attention to the animal population and household income levels in the region are the high number of patients (customers) they can treat (increasing their income) and reduced possibility of encountering problems regarding the fee they will charge after the interventions.

It was determined that the veterinarians participating in the survey covered the majority of the establishment costs of their practices from their own capital. In studies conducted in different times and provinces, the high rate of equity capital use by clinicians when establishing their practices supports the findings of the present study.[22,27] When the costs of the practices participating in the study were examined, the largest share was made up of medicine and food supplement costs. In the studies conducted by Kaygısız and Akdağ[22] and Erdoğan and Sarıözkan[27], the highest cost items were medicine, serum, and vaccine costs, which were confirmed by the findings of the present study, in which the highest income item of the practices examined in the study was the sale of medicines and food supplements. In the study conducted by Erdoğan and Sarıözkan[27] in Nevşehir province, the highest share in total income was obtained from artificial insemination (41.9%). In contrast to other studies, in the present study, the higher pharmaceutical and vaccine incomes than artificial insemination income can be attributed to the fact that the majority of farming enterprises in the region prefer natural insemination to artificial insemination. In addition, the fact that this practice is carried out by public institutions for veterinary practices that prefer artificial insemination suggests that this may cause private veterinary practices to have a low artificial insemination income.

The DEA revealed that 27 of the veterinary practices surveyed in Kars province were economically efficient and 3 veterinary practices were not efficient. High costs involving invoices, maintenance repair, and general administrative costs, which are common to all three, as well as costs of medical supplies (Practice 7, 10), depreciation (Practice 7, 10), rent (Practice 10, 14), artificial insemination (Practice 7), and employee (Practice 14), should be reduced to ensure that the three ineffective veterinary practices are effective. It has been concluded that the majority of veterinary practices operating in Kars

province are economically efficient, and those that are not efficient need to reduce some cost items according to DEA. The DEA used in this study guides veterinarians in determining whether practices are economically efficient or not and which cost items they need to reduce in order to be effective.

One of the most important criteria for determining the commercial performance of veterinary practices and comparing them with other practices is their level of economic activity. Various studies have been conducted on this subject in Europe and the USA. For example, according to the 2018 report of the European Federation of Veterinarians, the average monthly income of veterinarians in Europe was €3,300. The country with the highest income was Switzerland, where the average monthly income was around €6,800. For Türkiye, the average monthly income was reported as approximately € 3,000. According to 2019 reports from the US Bureau of Labor Statistics, the average hourly earnings of a veterinarian were US$ 45.9, and the monthly income was US$ 7,900.[30] In Kars, the monthly profit of actively working practices was US$ 2,765, while it was determined as US$ 1,266 in inactive veterinary practices.

As a conclusion, in recent years, there has been a significant increase in the number of veterinary practices in Kars province, parallel to Türkiye in general. It has been understood that practices encounter several problems both during the establishment phase and the operating period due to this increase. Among the problems experienced, it was observed that a lack of compensation for the service provided, unfair competition between practices, and ethical violations came to the fore. As a solution, it was concluded that it would be beneficial for veterinarians to give up different price practices and open a practice by eliminating their own deficiencies in the field of veterinary practice economics before establishing a practice. In addition, it is thought that the profitability of the clinics will increase by preventing problems and cost increases in the supply of vaccines, medicines and materials (the effect of TL depreciation and increasing exchange rates), general administration and rental expenses, debts and financial management problems. Providing low-interest loan opportunities and more effective work of professional organizations are important for the sustainability of veterinary practices.

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