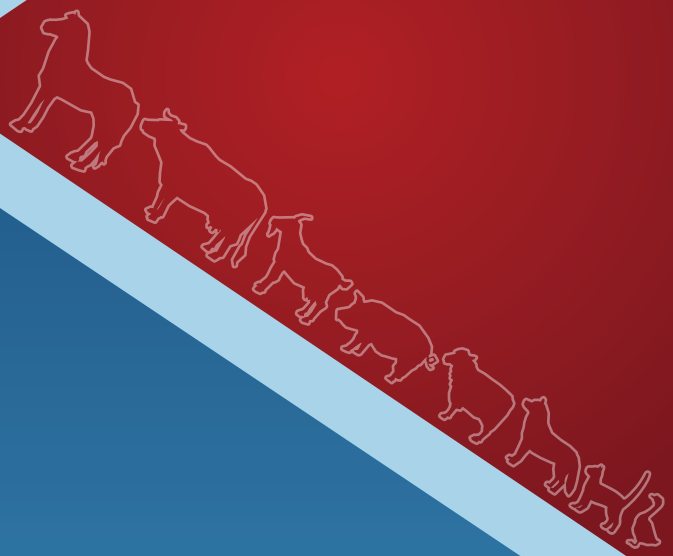
ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University



http://vetdergi.kafkas.edu.tr

Online Submission
http://submit.vetdergikafkas.org

Volume: 27
Issue: 5 (September- October)
Year: 2021

ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

Volume: 27
Issue: 5 (September - October)
Year: 2021

ISSN (Print): 1300-6045 **ISSN (Electronic): 1309-2251**

This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas, Kars - Turkey

This journal is indexed and abstracted in:

- Web of Science Core Collection: Science Citation Index Expanded (since 2007)
- Additional Web of Science Indexes: Essential Science Indicators - Zoological Record
- CABI - Veterinary Science Database
- DOAJ
- EBSCO - Academic Search Premier
- Elsevier - SCOPUS
- Elsevier - EMBASE
- Index Copernicus
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Phone: +90 474 2426807-2426836/5228 Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

Electronic Edition http://vetdergikafkas.org

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İÇİNDEKİLER (Contents)

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 543-545, 2021
DOI: 10.9775/2021.UNGA.editorial

EDITORIAL

Call for Emergency Action to Limit Global Temperature Increases, Restore Biodiversity, and Protect Health

WEALTHY NATIONS MUST DO MUCH MORE, MUCH FASTER

Lukoye ATWOLI [1] Abdullah H. BAQUI [2] Thomas BENFIELD [3] Raffaella BOSURGI [4] Fiona GODLEE [5] Stephen HANCOCKS [6] Richard HORTON [7] Laurie LAYBOURN-LANGTON [8 (*)] Carlos Augusto MONTEIRO [9] Ian NORMAN [10] Kirsten PATRICK [11] Nigel PRAITIES [12] Marcel GM OLDE RIKKERT [13] Eric J. RUBIN [14] Peush SAHNI [15] Richard SMITH [16] Nicholas J. TALLEY [17] Sue TURALE [18] Damián VÁZQUEZ [19]

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The UN General Assembly in September 2021 will bring countries together at a critical time for marshalling collective action to tackle the global environmental crisis. They will meet again at the biodiversity summit in Kunming, China, and the climate conference (COP26) in Glasgow, UK. Ahead of these pivotal meetings, we-the editors of health journals worldwide-call for urgent action to keep average global temperature increases below 1.5°C, halt the destruction of nature, and protect health.

Health is already being harmed by global temperature increases and the destruction of the natural world, a state of affairs health professionals have been bringing attention to for decades [1]. The science is unequivocal; a global increase of 1.5°C above the pre-industrial average and the continued loss of biodiversity risk catastrophic harm to health that will be impossible to reverse [2,3]. Despite the world's necessary preoccupation with covid-19, we cannot wait for the pandemic to pass to rapidly reduce emissions.

Reflecting the severity of the moment, this editorial appears in health journals across the world. We are united in recognising that only fundamental and equitable changes to societies will reverse our current trajectory.

The risks to health of increases above 1.5°C are now well established [2]. Indeed, no temperature rise is "safe." In the past 20 years, heat related mortality among people aged over 65 has increased by more than 50% [4]. Higher temperatures have brought increased dehydration and renal function loss, Dermatol ogical malignancies, tropical infections, adverse mental health outcomes, pregnancy complications, allergies, and cardiovascular and pulmonary morbidity and mortality [5,6]. Harms disproportionately affect the most vulnerable, including among children, older populations, ethnic minorities, poorer communities, and those with underlying health problems [2,4].

Global heating is also contributing to the decline in global yield potential for major crops, falling by 1.8-5.6% since 1981; this, together with the effects of extreme weather and soil depletion, is hampering efforts to reduce undernutrition [4]. Thriving ecosystems are essential to human health, and the widespread destruction of nature, including habitats and species, is eroding water and food security and increasing the chance of pandemics [3,7,8].

The consequences of the environmental crisis fall disproportionately on those countries and communities that have contributed least to the problem and are least able to mitigate the harms. Yet no country, no matter how wealthy, can shield itself from these impacts. Allowing the consequences to fall disproportionately on the most vulnerable will breed more conflict, food insecurity, forced displacement, and zoonotic disease-with severe implications for all countries and communities. As with the covid-19 pandemic, we are globally as strong as our weakest member.

How to cite this article?

Atwoli L, Baqui AH, Benfield T, Bosurgi R, Godlee F, Hancocks S, Horton R, Laybourn-Langton L, Monteiro CA, Norman I, Patrick K, Praities N, Olde Rikkert MGM, Rubin EJ, Sahni P, Smith R, Talley NJ, Turale S, Vázquez D: Call for emergency action to limit global temperature increases, restore biodiversity, and protect health. *Kafkas Univ Vet Fak Derg,* 27 (5): 543-545, 2021.
DOI: 10.9775/2021.UNGA.editorial

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Rises above 1.5°C increase the chance of reaching tipping points in natural systems that could lock the world into an acutely unstable state. This would critically impair our ability to mitigate harms and to prevent catastrophic, runaway environmental change [9,10].

Global Targets are not Enough

Encouragingly, many governments, financial institutions, and businesses are setting targets to reach net-zero emissions, including targets for 2030. The cost of renewable energy is dropping rapidly. Many countries are aiming to protect at least 30% of the world's land and oceans by 2030 [11].

These promises are not enough. Targets are easy to set and hard to achieve. They are yet to be matched with credible short and longer term plans to accelerate cleaner technologies and transform societies. Emissions reduction plans do not adequately incorporate health considerations [12]. Concern is growing that temperature rises above 1.5°C are beginning to be seen as inevitable, or even acceptable, to powerful members of the global community [13]. Relatedly, current strategies for reducing emissions to net zero by the middle of the century implausibly assume that the world will acquire great capabilities to remove greenhouse gases from the atmosphere [14,15].

This insufficient action means that temperature increases are likely to be well in excess of 2°C [16], a catastrophic outcome for health and environmental stability. Critically, the destruction of nature does not have parity of esteem with the climate element of the crisis, and every single global target to restore biodiversity loss by 2020 was missed [17]. This is an overall environmental crisis [18].

Health professionals are united with environmental scientists, businesses, and many others in rejecting that this outcome is inevitable. More can and must be done now-in Glasgow and Kunming-and in the immediate years that follow. We join health professionals worldwide who have already supported calls for rapid action [1,19].

Equity must be at the centre of the global response. Contributing a fair share to the global effort means that reduction commitments must account for the cumulative, historical contribution each country has made to emissions, as well as its current emissions and capacity to respond. Wealthier countries will have to cut emissions more quickly, making reductions by 2030 beyond those currently proposed [20,21] and reaching net-zero emissions before 2050. Similar targets and emergency action are needed for biodiversity loss and the wider destruction of the natural world.

To achieve these targets, governments must make fundamental changes to how our societies and economies are organised and how we live. The current strategy of encouraging markets to swap dirty for cleaner technologies is not enough. Governments must intervene to support the redesign of transport systems, cities, production and distribution of food, markets for financial investments, health systems, and much more. Global coordination is needed to ensure that the rush for cleaner technologies does not come at the cost of more environmental destruction and human exploitation.

Many governments met the threat of the Covid-19 pandemic with unprecedented funding. The environmental crisis demands a similar emergency response. Huge investment will be needed, beyond what is being considered or delivered anywhere in the world. But such investments will produce huge positive health and economic outcomes. These include high quality jobs, reduced air pollution, increased physical activity, and improved housing and diet. Better air quality alone would realise health benefits that easily offset the global costs of emissions reductions [22].

These measures will also improve the social and economic determinants of health, the poor state of which may have made populations more vulnerable to the covid-19 pandemic [23]. But the changes cannot be achieved through a return to damaging austerity policies or the continuation of the large inequalities of wealth and power within and between countries.

Cooperation Hinges on Wealthy Nations Doing More

In particular, countries that have disproportionately created the environmental crisis must do more to support low and middle income countries to build cleaner, healthier, and more resilient societies. High income countries must meet and go beyond their outstanding commitment to provide $100bn a year, making up for any shortfall in 2020 and increasing contributions to and beyond 2025. Funding must be equally split between mitigation and adaptation, including improving the resilience of health systems.

Financing should be through grants rather than loans, building local capabilities and truly empowering communities, and should come alongside forgiving large debts, which constrain the agency of so many low income countries. Additional funding must be marshalled to compensate for inevitable loss and damage caused by the consequences of the environmental crisis.

As health professionals, we must do all we can to aid the transition to a sustainable, fairer, resilient, and healthier world. Alongside acting to reduce the harm from the environmental crisis, we should proactively contribute to global prevention of further damage and action on the root causes of the crisis. We must hold global leaders to account and continue to educate others about the health risks of the crisis. We must join in the work to achieve environmentally sustainable health systems before 2040, recognising that this will mean changing clinical practice. Health institutions have already divested more than $42bn of assets from fossil fuels; others should join them [4].

The greatest threat to global public health is the continued failure of world leaders to keep the global temperature rise below 1.5°C and to restore nature. Urgent, society-wide changes must be made and will lead to a fairer and healthier world. We, as editors of health journals, call for governments and other leaders to act, marking 2021 as the year that the world finally changes course.

Competing Interests

We have read and understood BMJ policy on declaration of interests and FG serves on the executive committee for the UK Health Alliance on Climate Change and is a Trustee of the Eden Project. RS is the chair of Patients Know Best, has stock in UnitedHealth Group, has done consultancy work for Oxford Pharmagenesis, and is chair of the Lancet Commission of the Value of Death. None further declared.

Provenance and Peer Review

Commissioned; not externally peer reviewed.

This editorial is being published simultaneously in many international journals. Please see the full list here: *https://www.bmj.com/content/full-list-authors-and-signatories-climate-emergency-editorial-september-2021*

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 547-557, 2021
DOI: 10.9775/kvfd.2020.25171

RESEARCH ARTICLE

Lactation Milk Yield Prediction with Possibilistic Logistic Regression Analysis

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Article ID: KVFD-2020-25171 Received: 22.11.2020 Accepted: 24.08.2021 Published Online: 24.08.2021

Abstract

The logistic regression is a popular method to model the probability of a categorical outcome given as a dependent variable. However, the possibilistic logistic regression can be preferred instead of classical logistic regression when the dependent variable has uncertainity. The aim of this study is to use the possibilistic logistic regression on animal husbandry examining the theoretical foundations of the method based on fuzzy logic approach. A total of 90 cows were enrolled in the study and the average milk yield in 305 days was predicted by animal's weight, breed of the animal, age in lactation, number of milkings per day and the milking seasons of cows belonging to different breeds. The Mean Degree of Memberships (MDM) and the Mean of Squared Error (MSE) indices were calculated to decide the goodness of fit of the model. The index values were found as MDM=0.896 and MSE=4.871, respectively. It was shown that the model is fit and is succesfull to predict the average milk yield. It can be concluded that the model can provide the businesses on lactation milk yield production an efficient and accurate prediction results.

Keywords: *Fuzzy logistic regression, Lactation milk yield, Possibilistic odds, Minimization, Goodness-of-fit criteria*

Possibilistic Lojistik Regresyon Analizi İle Laktasyon Süt Verimi Tahmini

Öz

Lojistik regresyon analizi, bağımlı değişken olarak verilen özelliğin kategorilerini tahmin etmek için kullanılır. Ancak, bağımlı değişken belirsiz olduğunda klasik lojistik rgeresyon yerine posibilistik lojistik regresyon yöntemi tercih edilebilir. Bu çalışmanın amacı, süt sığırcılığında teorik altyapısı ile birlikte bulanık mantık yaklaşımı temelli posibilistik lojistik regresyon yöntemini kullanmaktır. Çalışmaya toplam 90 inekten elde edilen bilgiler dahil edildi ve hayvanın ağırlığı, hayvanın ırkı, hayvanın laktasyon yaşı, günlük sağım sayısı ve sağım mevsimi bilgileri kullanılarak ortalama süt verimi tahmin edildi. Modelin yeterliliğine karar verebilmek için ortalama üyelik derecesi (MDM) ve hata kareler ortalaması (MSE) indeks değerleri hesaplandı. İndeks değerleri sırasıyla MDM=0.896 ve MSE=4.871 olarak hesaplandı. Bu değerlere göre modelin uyumunun iyi olduğuna karar verildi. Bulgular, modelin laktasyon süt verimini tahminlemede etkin ve güvenilir sonuçlara sahip olduğunu göstermektedir.

Anahtar sözcükler: *Bulanık lojistik regresyon, Laktasyon süt verimi, Minimizasyon, Olabilirlik oranı, Uyum kriterleri*

INTRODUCTION

Classical set theory precisely determines the boundaries of sets and the properties of the elements belonging to the set. Each element in the set belongs to a set or not. Partial membership is never allowed. The boundaries of any set to be formed in natural life and the general characteristics of the elements that will form this set cannot always be determined precisely. Because of the uncertainties in the experiences, feelings and thoughts of the experts, their decisions contain an ambiguous language [1]. The question of which criteria and methods would be more accurate to use for more accurate estimation of the yield values of animals and for reliable diagnostic procedures causes disagreements among experts and leads to doubtful decisions. For example, experts who want to classify farm animals according to their milk, meat yields use linguistic expressions such as "low milk yield", "high meat yield", "sick or not sick". These linguistic expressions represent uncertainties belonging to experts [2,3]. It is not

How to cite this article?

Topuz D: Lactation milk yield prediction with possibilistic logistic regression analysis. *Kafkas Univ Vet Fak Derg,* 27 (5): 547-557, 2021. DOI: 10.9775/kvfd.2020.25171

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a correct choice to apply classical set theory in situations where the knowledge related to the experiences, feelings and thoughts of expert are valid [4]. Because these situations can be very difficult to model since they contain uncertainty. In addition, many scientists state that the source of the uncertainties is the inability to provide the sensitive devices required for measurement or the measurement of erroneous efficiency values due to the use of devices developed according to different criteria or misdiagnosis. In these cases, estimates, classifications and diagnosis processes become questionable because the assumptions required for the application of classical statistical methods cannot be provided [2]. Various theories are used to measure and evaluate uncertainty. Among those theories, probability theory and statistical methods are the preferred methods to model uncertainty [5]. However, many uncertainties encountered in daily life cannot be explained with randomness. Probability theory and statistical methods used to express uncertainties numerically may be inadequate in measuring nonrandom uncertainties [6]. These situations have led researchers to the idea that it is necessary to combine fuzzy sets and statistical theories. Therefore, fuzzy methods based on Zadeh's fuzzy set theory play a very important role in expressing qualitative expressions in human thought numerically in order to make more valid and reliable analyzes [1,7]. Fuzzy logistic regression analysis is one of the results of this combination and is used in cases where the assumptions of the classical logistic regression analysis method cannot be achieved. It can also be used if there is naturally uncertainty in observation values or relationships [8]. Since the approach takes into account "probability" rather than "probabilistic" errors, the error terms are distributed into fuzzy coefficients [9,10]. Studies conducted with fuzzy regression analysis approach are generally based on the applications of linear models. There are very few studies on nonlinear models.

The aim of this study was conducted to examine the theoretical foundations of possibilistic logistic regression analysis technique based on fuzzy logic approach and its application in animal husbandry, which can provide solutions to fuzzy situations. In order to estimate its model parameters, the applicability of the Diamond's Possibilistic method in animal husbandry proposed by Pourahmad et al was discussed for in this study.

Material and Methods

Ethical Statement

Data collection with the animal care and breeding practices from enterprise were used in this study in compatible with animal welfare rules stated in Article 9 in government law in Turkey (No.5996).

Materials

The material of the study consisted of 2005 milk yield records of 90 randomly selected cows from 220 cows of different breeds (Holstein Friesian, Brunette, South anatolian red, Cross breed) raised in a private farm. In order to estimate the likelihood values of average milk yield (Y_i) (kg) values in a lactation period with minimum error, values of independent variables such as the milk yield of cows in lactation (X_1), weight (X_2), breed (X_3), age in lactation (X_4), number of milkings per day (X_5) and milking season (X_6) were used as material. In addition, the Mean Degree of Memberships (MDM) and Mean of Squares Errors (MSE) indices were used to decide the adequacy of the model created.

Possibilistic Logistic Regression Analysis Model

Logistic regression model is the most commonly used regression model in cases where the dependent variable is categorical [11]. Logistic regression analysis is preferred when the relevant dependent variable consists of categories such as "low efficiency - high efficiency", "sick - not sick". Fuzzy logistic regression analysis approach is a regression method based on fuzzy set theory, and it is a fuzzy approach used to analyze the uncertainties in the natural structure of the data which belongs to the dependent variable [11,12]. The approach is a regression method based on fuzzy set theory used in cases where classical logistic regression analysis assumptions cannot be fulfilled or the data is fuzzy due to its nature [8]. It can also be used in situations where observation values or relationships between variables are uncertain [7,13-15]. The linear regression is not applicable to some situations such as when there is a small data set, vagueness in the relationship between the predictors and response variables, and inaccuracy or distortion introduced by linearization. Therefore, fuzzy regression is generally performed to complement those situations and problems [6]. Logistic regression modeling is a nonlinear statistical method used to model a categorical response variable based on some covariates [1]. In fuzzy field, fuzzy logistic regression for binary base response is also defined. Studies on this topic can be categorized into possibilistic methods and distance-based methods. A common viewpoint which is applied by Nagar and Srivastava [4] simultaneously, used a possibilistic-based approach to investigate a certain fuzzy logistic regression model. In fact, they used this approach to predict the oral cancer based on some real data sets.

Since the estimated values of the dependent variable are calculated by probability, the equations belonging to the fuzzy logistic regression model showing the possibilistic value are as follows [4,9,11,13-16];

$$\widetilde{W}_j = \ln\left\{\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right\} = \widetilde{A}_0 + \widetilde{A}_1 x_{i1} + \widetilde{A}_2 x_{i2} + \widetilde{A}_3 x_{i3} + \cdots + \widetilde{A}_{p-1} x_{i(p-1)} \quad (1)$$

Here; It is a data set which is $X_i = \left[x_{i1}, x_{i2}, .., x_{i(p-1)}\right] i = 1, \ldots, n$ and it is the observation vector of independent variables consisting of precise values such as milk yield, weight, race, age in lactation, daily milking number and milking season of i^{th} cow. Each independent variable observation is expressed as $x \in X$

(i: 1,2,3,..,p (p, number of independent variables). $\tilde{\mu}_i$: is the value indicating that the i[th] unit can have the desired property for the relevant dependent variable as $\tilde{\mu}_i = \text{Poss}(Y_i = 1)$ [6,12,17,18].

$\widetilde{A}_i = \left[\widetilde{A}_0 \text{ ve } \widetilde{A}_1, \widetilde{A}_2, \ldots \widetilde{A}_i \ldots, \widetilde{A}_{p-1}\right]$ is the coefficient values of the independent variables in the function, represented by $\widetilde{A}_i = [a_i^c, a_i^s]$ [2,19,20]. a_i^c: is the mode value representing the center of the fuzzy coefficients and is in the form of $a_i^c = [a_0^c, a_1^c, \ldots, a_n^c]$. a_i^s: is the value showing the spread of fuzzy coefficients and it is in the form of $a_i^s = [a_0^s, a_1^s, \ldots, a_n^s]$. Hence each coefficient is defined by $\widetilde{A}_0 = [a_0^c, a_0^s]$, $\widetilde{A}_1 = [a_1^c, a_1^s], \ldots, \widetilde{A}_6 = [a_6^c, a_6^s]$ [3,20]. $\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}$: shows the probability that the i[th] case will have the property considered for each fuzzy case, and is called possibilistik odss [9,15]. $\widetilde{W}_j = \ln\left\{\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right\}$: estimated fuzzy output value [4,11].

Estimation of Fuzzy Coefficients

The approach suggested by Pourahmad et al.[14] based on Diamond's likelihood (possibilistic) approach was used [3,21-23]. The main purpose is to minimize the total uncertainty of the model by minimizing the total spread of fuzzy coefficients. $\widetilde{A}_i = (a_j^c, s_j^L, s_j^R)$: $s_j^L = s_j^R = s_j$ $j = 0,1, \ldots, p-1$ the coefficient values are assumed as triangular fuzzy numbers because they represent measurement errors and general uncertainties. Fuzzy output $\widetilde{W}_j (j = 1, .., m)$ values estimated by equation (1) according to fuzzy arithmetic operations are also modeled as in equation (2) because they are fuzzy numbers with symmetrical triangular properties [19],

$$\widetilde{W}_j = f(x, \widetilde{A}) = [f_i^c(x), f_i^s(x)] = \left[f_i^c(x), f_{is}^L(x), f_{is}^R(x) \right]_T \quad (2)$$

Here, $f_i^s(x)$: is the spread of the fuzzy logistic regression approach and is as $f_i^s(x) = [a_0^s + a_1^s x_{i1} + a_2^s x_{i2} +, \ldots, +a_n^s x_{in}]$. $f_i^c(x)$ is the mode value of the fuzzy logistic regression approach as in equation (3) [24].

$$f_i^c(x) = a_0^c + a_1^c X_{i1} + \cdots + a_{p-1}^c X_{in},\ F_{is}^L(x) = s_0^L + s_1^L X_{i1} + \cdots + s_{p-1}^L X_{in},\ f_{is}^R(x) = s_0^R + s_1^R X_{i1} + \cdots + s_{p-1}^R X_{in} \quad (3)$$

The $\widetilde{W}_j$ membership function of the dependent variable, estimated by the fuzzy logistic regression analysis method, can be shown as in equation (4);

$$\widetilde{W}_j(\widetilde{Y}_j) = \begin{cases} 1 - \frac{f_i^c(x) - \widetilde{Y}_j}{f_{is}^L(x)}, & f_i^c(x) - f_{is}^L(x) \le \widetilde{Y}_j \le f_i^c(x), \\ 1 - \frac{\widetilde{Y}_j - f_i^c(x)}{f_{is}^R(x)}, & f_i^c(x) < \widetilde{Y}_j \le f_i^c(x) + f_{is}^R(x), \\ 0 & \text{otherwise} \end{cases} \quad (4)$$

If the $\widetilde{W}_j = f(x, \widetilde{A}) = [f_i^c(x), f_i^s(x)]$ $\widetilde{A}_i = [a_i^c, a_i^s]$: $s_i^L = s_i^R = s_i$ $j = 0,1, \ldots, p-1$ values are equal to each other. This state is expressed as an [15,16,19].

$$\widetilde{W}_j = [f_i^c(x), f_i^S(x)] = f_{is}^L(x) = f_{is}^R(x) = f_i^S(x) \ (i = 1,2,3,\ldots,m) \quad (5)$$

These values will be obtained as a fuzzy number with symmetric triangular property equal to each other. So, after estimating the model coeficients, we can determine the membership function of the possibilistic odds, $f(\widetilde{W}_j) = \exp\left[\widetilde{W}_j(x)\right], x > 0$ as follows; membership function is created as in equation (6) [4,11,15,16].

$$\exp\left[\widetilde{W}_j(x)\right] = \widetilde{W}_j(\ln(x)) = \begin{cases} 1 - \frac{f_i^c(x) - \ln(x)}{f_{is}^L(x)}, & f_i^c(x) - f_i^L(x) \le \ln(x) \le f_i^c(x), \\ 1 - \frac{\ln(x) - f_i^c(x)}{f_{is}^R(x)}, & f_i^c(x) < \ln(x) \le f_i^c(x) + f_i^R(x). \end{cases} \quad (6)$$

These assumptions are as follows:

1-The real lactation milk yield value ($\widetilde{Y}_j$) of each cow should be estimated from the ($\widetilde{W}_i$) value estimated with the fuzzy approach, with a membership degree of the least turbidity tolerance coefficient (h) value$\left(\widetilde{W}_j(\widetilde{Y}_j) \ge h\right)$ [13]. Here $\widetilde{Y}_j$: is the observed value of the dependent (response) variable, which is a definite number, and is as
$\widetilde{Y}_j = \ln\left\{\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right\}, h \in (0.1)$ [11,25].

2- The objective function minimizing the ($\widetilde{W}_i$) spread of the fuzzy output values to be estimated is as in equation (7) [14,15,24];

$$Z = [m(s_0^L + s_0^R)] + \sum_{j=1}^{p-1}\left[\left(s_j^L + s_j^R\right)\sum_{i=1}^{n} xij\right] \quad (7)$$

Here, n: is the number of observations regarding the dependent variable. j: the number of independent variable. x_{ij}: i[th] observation value of the j[th] independent variable. The number of observations n determines the number of constraints because a range is estimated for $\widetilde{Y}_j$ by approaching from the left and right. The constraint number for each estimated $\widetilde{W}_i$ value should be 2xn [21]. Using Equation (6), the constraint limitation of each situation $\widetilde{W}_j(\widetilde{Y}_j) \ge h, j = 1,2,\ldots,n$ is as in equations (8) and (9) [5,15]:

$$1 - \frac{f_i^c(x) - \widetilde{Y}_j}{f_{is}^L(x)} \ge h \Rightarrow (1-h) f_{is}^L(x) - f_i^c(x) - \ge \widetilde{Y}_j \qquad i = 1,2,3,..,n$$
$$1 - \frac{\widetilde{Y}_j - f_i^c(x)}{f_{is}^R(x)} \ge h \Rightarrow (1-h) f_{is}^R(x) + f_i^c(x) \ge \widetilde{Y}_j \qquad i = 1,2,3,..,n \quad (8)$$

By rearranging (8) Equality, we can create constraints [12] for sample data sets as in equation (9)

$$(1-h)s_o^L + (1-h)\sum_{j=1}^{p-1}(s_i^L x_{ij}) - a_o^c - \sum_{j=1}^{p-1}(a_i^c x_{ij}) \ge -\widetilde{Y}_j \ \forall i, i = 1,2,\ldots,n$$
$$(1-h)s_o^R + (1-h)\sum_{j=1}^{p-1}(s_j^R x_{ij}) + a_o^c + \sum_{j=1}^{p-1}(a_i^c x_{ij}) \ge \widetilde{Y}_j \quad \forall i, i = 1,2,\ldots,n \quad (9)$$

The first constraint is for calculating the mean and left and right spreads of each coefficient, while the second constraint is for minimizing the objective function based on linear programming (Equation 8) [5,7,26,27].

Goodness of Fit Test Criterion for Fuzzy Regression Models

Fuzzy logistic regression attempts to model and predict

the possibility of success based on fuzzy covariates. To appraise the goodness of fit of the fuzzy logistic regression, we introduce some criteria. These are "Mean degree of membership (possibilistic odds) (MDM), "Mean of Squares Errors (MSE) [16].

Mean Degree of Memberships (MDM)

The average degree of membership is a criterion similar to the coefficient of expression (R^2), which indicates how much of the observed change in the dependent variable is explained by the observed change in the independent variables [22]. With the help of an average membership level of 10;

$$\mathrm{MDM} = \frac{1}{m}\sum_{i=1}^{n}\widetilde{W}_j\left(\widetilde{Y}_j\right) = \frac{1}{m}\sum_{i=1}^{n}\exp\left[\widetilde{W}_j\left(\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right)\right] \quad (10)$$

can be calculated [27]. Here; $\widetilde{Y}_j$: is the observed value of the dependent variable, which is an absolute bivalent number for the j^{th} case. $\widetilde{W}_j$: is the natural logarithm of the odds of possibilistic estimated as a fuzzy number. $\exp\left[\widehat{\widetilde{W}}_i\left(\frac{\mu_i}{[1-\mu_i]}\right)\right] = \frac{\mu_i}{[1-\mu_i]}$: i^{th} is the estimated value of the possibility of the odds which belongs to the i^{th} observation. By the help of Equation (11);

$$\exp\left[\widetilde{W}_j\left(\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right)\right] = 1 - \frac{\left(\widetilde{Y}_j - f_i^c(x)\right)}{f_i^s(x)} \text{ veya } 1 - \frac{\left(f_i^c(x) - \widetilde{Y}_j\right)}{f_i^s(x)} \quad (11)$$

can be calculated. It is desirable that the average of the calculated membership degrees is close to 1. Average membership degree takes values between 0 and 1 [16,26,27].

Mean Square Error Test Criteria (MSE)

It is an index used to evaluate the goodness of fit. The closer the predicted value is to the observed value, the stronger the model's power to predict real situations [17]. The calculation formula is as Equation (12) [9,28];

$$\mathrm{MSE} = \frac{1}{m}\sum_{i=1}^{n}\left[\mathrm{defCoG}\left\{\exp\left[\widetilde{W}_j\right]\right\} - \left(\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right)\right]^2 \quad (12)$$

Here; $\mathrm{defCoG}\left\{\exp\left[\widetilde{W}_j\right]\right\}$, $\exp\left[\widetilde{W}_j\left(\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right)\right]$ is the estimated value of the possibilistic odds for defuzzification. Since the estimated fuzzy values will have to be converted to numerical (exact, net) values, the clarification process using the center of Gravity defuzzication method, which is one of the clarification methods, is determined by equation (13) and,

Statistical Analysis

The statistical analyses were performed by Microsoft Office EXCEL 2016 (R) and LINGO 16.0 softwares. The MDM and MSE values were calculated to decide the adequecy of the model.

Results

Step 1. Experts and producers may be suspicious of the measurements made because sensitive devices required for the measurement of average milk yield values in a lactation period cannot be provided or due to measurement errors. In such cases, they cannot represent the average milk yield value of each animal by one of the two dependent response categories. In other words, since the observed values of the dependent variable do not represent a certain situation, it cannot be stated that they belong to any category as 0 or 1. Therefore, due to the uncertainty in the dependent variable, the probability that the i^{th} animal belongs to the category 1 $(E(Y) = P(Y = 1) = p)$ and its odds ratio $\left\{\frac{p_i}{[1-p_i]}\right\}$ cannot be calculated [13,29].

For this purpose, independent variables were determined as milk yield of cows in lactation (X_1), weight (X_2), race (X_3), age at lactation (X_4), daily milking number (X_5) and milking season (X_6). The dependent variable is whether the average milk yield (Y_i) (kg) values in a lactation period have normal yield values according to the mentioned independent variables. Expert opinion was taken to determine the likelihood of lactation milk yield values. In order to determine the likelihood of lactation milk yield values, we defined a measure such as $\tilde{\mu}_i \in$ { No high milk yielding cow=0, High milk yielding cow=1}, as the degree of consistency (probability measure) of the known characteristic for each turbid state representing the average milk yield (Y_i) (kg) values (LSV) in a lactation period (LSV <3000 kg = 0, LSV≥3000 kg = 1). According to the defined measure $\tilde{\mu}_i$: is the probability of whether the animal is a low or high milk yield one or not, instead of 0 and 1, which replace the observed value of the dependent variable and the degree of membership indicating the likelihood of belonging to any category in the $\mu_i \in R: 0 \le \tilde{\mu}_i \le 1$ [12,16,17,26] range i =1,2,.., 90. The complement of each situation is $1 - \tilde{\mu}_i \in R: 0 \le 1 - \tilde{\mu}_i \le 1$. The $\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}$ value indicating the ratio of the probability of having a certain property of the related fuzzy observation and not being possible is called "possibilistic odds" [4,23].

In order to apply the fuzzy logistic regression analysis method, the possibility of the i^{th} case to have this related property due to the uncertainty in the dependent variable is expressed as $\tilde{\mu}_i = \mathrm{Poss}(Y_i = 1)$[4,10,12]. We calculated the probability ratios by applying logarithmic transformations for the values of the binary dependent variables according to different significance levels.

$$\mathrm{def\,CoG}\left\{\exp\left[\widetilde{W}_j\right]\right\} = \frac{\int_{\exp\left(f_i^c(x)\right)}^{\exp\left(f_i^c(x)\right)} x\left(1 - \frac{f_i^c(x) - \ln(x)}{f_i^s(x)}\right)dx + \int_{\exp\left(f_i^c(x)\right)}^{\exp\left(\left(f_i^c(x)+f_i^s(x)\right)\right)} x\left(1 - \frac{\ln(x) - f_i^c(x)}{f_i^s(x)}\right)dx}{\int_{\exp\left(f_i^c(x)-f_i^s(x)\right)}^{\exp\left(\left(f_i^c(x)\right)\right)} x\left(1 - \frac{f_i^c(x) - \ln(x)}{f_i^s(x)}\right)dx + \int_{\exp\left(f_i^c(x)\right)}^{\exp\left(\left(f_i^c(x)+f_i^s(x)\right)\right)} x\left(1 - \frac{\ln(x) - f_i^c(x)}{f_i^s(x)}\right)dx} \quad (13)$$

can be calculated [15,28].

Within the same logic, we calculated the measurement value

Table 1. The number of fuzzy triangle $\tilde{\mu}_j$ and its corresponding $\tilde{Y}_j$ transformations

No	Linguistic Expression of the Fuzzy Binary Dependent Variable (Y_i)	$\tilde{\mu}_i$	$\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}$	$\tilde{Y}_j = \ln\left\{\frac{\tilde{\mu}_i}{[1-\tilde{\mu}_i]}\right\}$
	0	(0.01; 0.05)	(0.01; 0.05)	(-4.605; -2.995)
	1	(0.95; 0.99)	(19; 99)	(2.944; 4.595)
1	1	0.283	0.395	-0.9281
2	1	0.142	0.166	-1.7938
.	.	.	.	.
89	1	0.584	1.404	0.3392
90	0	0.029	0.030	-3.5110

** Not having a high milk yield cow = 0, High milk yielding cow = 1*

corresponding to $\tilde{\mu}_i$ and the probability values of 90 cows as a triangular fuzzy number ($\tilde{Y}_j$) with the symmetric triangular membership function, as in *Table 1*.

We used these calculated fuzzy values as output observation values.

Step 2. In order to minimize the uncertainties of independent variables affecting the average milk yield in a lactation period at the level of h=0.5, constraint values from $\tilde{Y}_1 = \log_e\left\{\frac{0.2833}{[1-0.2833]}\right\} = -0.9281$ to $\tilde{Y}_{90} = \log_e\left\{\frac{0.0290}{[1-0.0290]}\right\} = -3.5110$ were calculated and 180 (90*2) constraint matrices were created as in equation (14):

$$\text{MIN} = 90 * a_0^s + 46903 * a_1^s + 7799 * a_2^s + 28 * a_3^s + 1178 * a_4^s + 51 * a_5^s + 9 * a_6^s$$

$$\tilde{Y}_1 = \begin{bmatrix} -0.5 * a_0^s - 3720 * a_1^s - 563 * a_2^s - 0 * a_3^s - 78 * a_4^s - 3 * a_5^s - 1 * a_6^s + a_o^c + 3720 * a_1^c + 563 * a_2^c + 0a_3^c + 78 * a_4^c + 3 * a_5^c + 1 * a_6^c <= -0.928; \\ 0.5 * a_0^s + 3720 * a_1^s + 563 * a_2^s + 0 * a_3^s + 78 * a_4^s + 3 * a_5^s + 1 * a_6^s + a_o^c + 3720 * a_1^c + 563 * a_2^c + 0 * a_3^c + 78 * a_4^c + 3 * a_5^c + 1 * a_6^c >= -0.928; \end{bmatrix}$$

. . (14)

$$\tilde{Y}_{90} = \begin{bmatrix} -0.5 * a_0^s - 786 * a_1^s - 176 * a_2^s - 3 * a_3^s - 41 * a_4^s - 2 * a_5^s - 0 * a_6^s + a_o^c + 786 * a_1^c + 176 * a_2^c + 3 * a_3^c + 41 * a_4^c + 2 * a_5^c + 0 * a_6^c <= -3.511; \\ 0.5 * a_0^s + 386 * a_1^s + 176 * a_2^s + 3 * a_3^s + 41 * a_4^s + 2 * a_5^s + 0 * a_6^s + a_o^c + 386 * a_1^c + 176 * a_2^c + 3 * a_3^c + 41 * a_4^c + 2 * a_5^c + 0 * a_6^c >= -3.511; \end{bmatrix}$$

@FREE(C1); @FREE(C2); @FREE(C3); @FREE(C4); @FREE(C5); FREE(C6); END

The first six elements in each row of the constraint matrices show the center (a_i^c) values, and the last six elements show the spread (a_i^S) values.

Step 3. The possibilistic model to be created at the h = 0.5 level is

$$\tilde{W}_j = \tilde{A}_0 + \tilde{A}_1 * \text{LSV} + \tilde{A}_2 * \text{HA} + \tilde{A}_3 * \text{HI} + \tilde{A}_4 * \text{HLY} + \tilde{A}_5 * \text{GSS} + \tilde{A}_6 * \text{SM},\ j = 1,2,\ldots,90$$

To estimate the $\tilde{A}_i$, i = 0,1,...,6, coefficients which belong to the model, Diamond's possibilistic method, which is based on the linear programming method, was used [23]. The values in *Table 2* are calculated by using the constraint matrices in the equation (14) created with this logic approach in "Lingo 16.0" software [16,21].

Studies have determined that changing the value of h does not affect the center of the coefficients (a_i^c) but affects the spread (a_i^s) and the value of the objective function (Z) [5].

Step 4. The fuzziness of the model to be created at the level of h = 0.5, considering the total values of the variables and the dispersion values, was calculated as in the Equation (15) [21,28].

Table 2. Coefficient values of fuzzy logistic regression approach

Variables	$\widetilde{A}_i = (a_i^c; a_i^s)$			Classical Logistic Regression	
	$\widetilde{A}_i$	a_i^c	a_i^s	$\widehat{\beta}$	Std. Error
Constant	$\widetilde{A}_0$	3.6932	0.000	-0.4225	0.364
LSV(X_1)	$\widetilde{A}_1$	0.0005	0.000	-1.3646	0.859
HA(X_2)	$\widetilde{A}_2$	0.0050	0.000	0.0782	2.078
HI(X_3)	$\widetilde{A}_3$	-1.2546	0.523	1.6721	1.052
HLY(X_4)	$\widetilde{A}_4$	-0.0228	0.069	-0.0272	2.194
GSS(X_5)	$\widetilde{A}_5$	-2.6493	0.000	2.9756	0.157
SM(X_6)	$\widetilde{A}_6$	0.3736	0.000	-1.7321	0.675

LSV: lactation milk yield, HA: animal's weight, HI: breed of the animal (Holstein Friesian = 0, brunette = 1, south anatolian red = 2, cross breed = 3), HLY: the animal's age at lactation (Moon), GSS: number of milkings per day, SM: milking season (winter and autumn = 0, spring and summer = 1)

$$Z = \left[90\, a_0^S + a_1^S \sum_{i=1}^{90} x_{1i} + a_2^S \sum_{i=1}^{90} x_{2i} + a_3^S \sum_{i=1}^{90} x_{3i} + a_4^S \sum_{i=1}^{90} x_{4i} + a_5^S \sum_{i=1}^{90} x_{5i} + a_6^S \sum_{i=1}^{90} x_{6i}\right] \tag{15}$$

$$Z = [90 * 0.0 + 46903 * 0.0 + 7799 * 0.0 + 28 * 0.5235 + 1178 * 0.0693 + 51 * 0.0 + 9 * 0.0]$$

$Z_{1X12} = 96.293$. This objective function, calculated in 90x14 dimensions, is minimized by limiting it to 180 (90 observations × 2) matrix of coefficients.

Step 5. The best fuzzy logistic regression analysis equation created at Z = 96.293 fuzziness value is calculated as in the Equation (16).

$$\begin{aligned}\widetilde{W}_j = {} & (3.6932; 0.0) + (0.0005; 0.0) * X_1 + (0.0050; 0.00) * X_2 + (-1.2546; 0.523) * X_3 \\ & + (-0.0228; 0.069) * X_4 + (-2.6493; 0.0) * X_5 + (0.3736; 0.0) * X_6\end{aligned} \tag{16}$$

With Equation (16), it is tried to determine whether each cow is a high milk producing or low milk producing cow with the constraint lines of the independent variable values used. The possibilistic probability values of the lactation milk yield values to be estimated in these unclear situations are a fuzzy number with a symmetrical triangular feature. When we want to calculate the posibilistic odds of ratio of the average milk yield value of the number one cow in a lactation period, the relevant data are applied and calculated in equation (17);

$$\begin{aligned}\widetilde{W}_1 = \left[\ln\left(\frac{\tilde{\mu}_1}{[1-\tilde{\mu}_1]}\right)\right] = {} & (3.6932; 0.0) + (0.0005; 0.0) * 3720 + (0.0050; 0.0) * 563 + (-1.2546; 0.523) * 0 + (-0.0228; 0.069) * 78 \\ & + (-2.6493; 0.0) * 3 + (0.3736; 0.0) * 1 \\ = {} & (3.6932; 0.0) + (1.86; 0.0) + (2.815; 0.00) + (0.00; 0.00)_T + (-1.7784; 5.382) + (-7.9479; 0.0) + (0.3736; 0.0) = (-1.9850; 5.382)_T\end{aligned} \tag{17}$$

Here, $\widetilde{W}_1 \cong (-1.98;\ 5.38)_T$ is the natural logarithm of the possibilistic odds of ratio of the average milk yield value of the number one cow in a lactation period and is -1.98.

Step 6. For each cow, calculations were made as in equation (17) and the values in *Table 3* were obtained.

Step 7. In order to calculate the predicted value of the possibilistic odds of ratio calculated for each cow in the sixth step, extension principle in equation (6) is used. For example, the posibilistic odds of ratio of the average amount of milk i lactation period belonging to number one cow ($\exp\left[\widetilde{W}_1\right] = \frac{\tilde{\mu}_1}{[1-\tilde{\mu}_1]}$) $= \widetilde{W}_1 \left[\ln \frac{x}{1-x}\right]$ is calculated as in equation (18) [20].

Table 3. Some calculated fuzzy statistical values of sample data set

No	$\widetilde{Y}_j = \ln\left\{\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right\}$	Estimated Values with Fuzzy Approach: $\widetilde{W}_j(x)$	
		$f_i^C(x)$	$f_i^S(x)$
1	-0.928	-1.98	5.38
2	-1.794	-1.52	5.93
.	.	.	.
89	0.339	-0.58	3.83
90	-3.511	-5.03	4.40

$$\exp[\widetilde{W}_1] = \left\{ \begin{array}{l} 1 - \dfrac{-1.98 - \ln\left(\frac{x}{1-x}\right)}{5.38}, -7.36 \le \ln\left(\frac{x}{1-x}\right) \le -1.98\ [0.0063 \le x \le 0.138] \\ \\ 1 - \dfrac{\ln\left(\frac{x}{1-x}\right) + (-1.98)}{5.38}, -1.98 < \ln\left(\frac{x}{1-x}\right) \le 3.40 [0.138 \le x \le 29.96] \end{array} \right\} \quad (18)$$

Possibilistik odds of having a high milk yield cow according to the fuzzy observation values of cow number one was calculated as 0.138.

Step 8. Since the calculated $\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}$ rate values can take values between 0 and $+\infty$, logit transformation is made by taking the natural logarithms of the odds values (logit is made by taking the natural logarithm of the odds value of an event). As a result of the transformation, the nonlinear logistic regression function is transformed into a linear symmetric function by ensuring that the limits of $\widetilde{Y}_j = \ln\left\{\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right\}$ values are taken from the range $(0, +\infty)$ to the limit $(-\infty, +\infty)$ [28]. The estimated fuzzy likelihood $(\widehat{P}_i)$ values are calculated by applying transformation to the estimated posibilistic odds ratio values. Because $0.138 = \frac{\hat{\mu}_1}{[1-\hat{\mu}_1]}$ for number one cow, It has been calculated as $\hat{\mu}_1 = 0.138 - 0.138 * \hat{\mu}_1 \rightarrow \hat{\mu}_1 + 0.138 * \hat{\mu}_1 = 0.138 \rightarrow 0.138 * \hat{\mu}_1 = 0.138 \rightarrow \hat{\mu}_1 = \frac{0.138}{1.138} = 0{,}121.$

This calculated value is the high milk yield likelihood value of number one cow and it was calculated as $\exp[\widetilde{W}_1] = \widetilde{W}_1\left[\ln\frac{x}{1-x}\right] = \widetilde{W}_1(-1.98) = \exp(\widetilde{W}_1(0.395)$. This calculated value is explained with the $\exp(\widetilde{W}_1(0.395)$ =0.121 possibilistic that the number one cow is the normal milk producing cow. And for the cow no 90, it is calculated as $\exp[\widetilde{W}_{90}]$;

$$\exp[\widetilde{W}_{90}] = \left\{ \begin{array}{l} 1 - \dfrac{-5.03 - \ln\left(\frac{x}{1-x}\right)}{4.40}, -5.03 - 4.40 \le \ln\left(\frac{x}{1-x}\right) \le -5.03\ [e^{-5.03-4.40} \le x \le e^{-5.03}] \\ \\ 1 - \dfrac{\ln\left(\frac{x}{1-x}\right) + (-5.03)}{4.40}, -5.03 < \ln\left(\frac{x}{1-x}\right) \le -5.03 + 4.40 [e^{-5.03} \le x \le e^{-5.03+4.40}] \end{array} \right\} \quad (19)$$

$\exp[\widetilde{W}_{90}] = (-3.51) = \exp(\widetilde{W}_{90}(0.03).\ \exp[0.395] \ge h = 0.5$ is indicated by the value of tolerance coefficient (h). This calculated value is the predicted value of the possibilistic odds of the cow numbered ninety to be a high milk producing cow. Another important feature of the fuzzy logistic regression analysis approach is that the probability of probability (posibilistic odds) value can be predicted when a new cow other than the cows used is added to the study or business. For example, suppose that the 91th low milk yield cow with an average lactation milk yield of 2850 kg and $x_2 = 630,\ x_3 = 0,\ x_4 = 41,\ x_5 = 2,\ x_6 = 0$ will be included in the enterprise. By applying these values at equation (16), possibilistic odds ratio of average milk yield in a lactation period (Possibilistic odds) is calculated as:

$$\begin{aligned} \widetilde{W}_{91} = &\left[\log_e\left(\frac{\widetilde{\mu}_{91}}{[1-\widetilde{\mu}_{91}]}\right)\right]; (3.6932; 0.0) + (0.0005; 0.0) * 2850 + (0.0050; 0.00) * 630 + \\ &(-1.2546; 0.523) * 0 + (-0.0228; 0.0693) * 41 + (-2.6493; 0.0) * 2 + (0.3736; 0.0) * 0 \\ &= (2.143; 2.866)_T \cong (2.14; 2.87)_T \end{aligned} \quad (20)$$

According to the fuzzy observation values of the newly added cow, the value showing the possibilistic odds of a low milk yield cow can be calculated as follows: $\exp[\widetilde{W}_{91}]$;

$$\exp[\widetilde{W}_{91}] = \left\{ \begin{array}{l} 1 - \dfrac{2.14 - \ln\left(\dfrac{x}{1-x}\right)}{2.87}, -0.73 \le \ln\left(\dfrac{x}{1-x}\right) \le 2.14 \; [0.482 \le x \le 8.49] \\ \\ 1 - \dfrac{\ln\left(\dfrac{x}{1-x}\right) + (2.14)}{2.87}, 2.14 < \ln\left(\dfrac{x}{1-x}\right) \le 5.01 \;\; [8.49 \le x \le 149.90] \end{array} \right\} \quad (21)$$

It is seen that the posibilistic odds value of this newly added cow is calculated as 8.49. This value is the value that indicates the ratio of the probability of cow 91 being a low-yielding animal to the probability of not being low milk yield. With the possibility of 0.894, it can be said that the newly added cow no 91 has low milk yield after performing the relevant reverse conversions. Calculations for each other cow were made in this way and the values in *Table 4* were obtained.

Table 4. *Observed and predicted fuzzy statistical values of sample data set*

No	$\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}$	$\widetilde{Y}_j = \ln\left\{\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right\}$	Estimated Values with Fuzzy Approach: $\widetilde{W}_j(x)$		The Predicted Values of Probability of Average Milk Yield Values According to the Expansion Principle: $\exp\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right] = \widetilde{W}_j(\widetilde{Y}_j)$
			$f_i^C(x)$	$f_i^S(x)$	
1	0.395	-0.928	-1.98	5.38	$\widetilde{W}_1(-0.928) = \exp(\widetilde{W}_1(0.395)$
2	0.166	-1.794	-1.52	5.93	$\widetilde{W}_2(-1.794) = \exp(\widetilde{W}_2(0.166)$
.	.	.	.		. . .
89	1.404	0.339	-0.58	3.83	$\widetilde{W}_{89}(0.339) = \exp(\widetilde{W}_{89}(1.404)$
90	0.030	-3.511	-5.03	4.40	$\widetilde{W}_{90}(-3.511) = \exp(\widetilde{W}_{90}(0.030)$

Step 9. In order to calculate the "Average Degree of Membership (MDM)" value of the fuzzy logistic regression analysis model in Equation (16), the average milk yield value for each cow in a lactation period and the estimated turbid output (mean milk yield) value are used in equations (11) and (12). Membership degrees of the predicted possibilistic odds of average milk yield values in a lactation period for each cow are calculated as:

$$\widetilde{W}_1(x) = \left\{ \begin{array}{l} 1 - \dfrac{-1.98 - \ln\left(\dfrac{x}{1-x}\right)}{5.38} \\ \\ 1 - \dfrac{\ln\left(\dfrac{x}{1-x}\right) + (-1.98)}{5.38} \end{array} \right\} \Rightarrow \widetilde{W}_1(x) = 1 - \frac{(-0.93) - (-1.98)}{5.38} = \widetilde{W}_1(-0.93) \cong 0.80 \quad (22)$$

And the values in *Table 5* are obtained.

Table 5. Membership degrees of observed and predicted turbid output values of lactation milk yields

No	$\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}$	$\widetilde{Y}_j = \ln\left\{\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right\}$	Estimated Values with Fuzzy Approach: $\widetilde{W}_j(x)$		$\exp\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]$
			$f_i^C(x)$	$f_i^S(x)$	
1	0.395	-0.928	-1.98	5.38	0.80
2	0.166	-1.794	-1.52	5.93	1.00
.	.	.	.	.	.
89	1.404	0.339	-0.58	3.83	0.76
90	0.030	-3.511	-5.03	4.40	0.65
Total					80.64
MDM					0.896

The $\exp\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]$ value calculated in Table 5 is the estimated membership degree values of fuzzy numbers in the symmetrical triangular property structure. That is, $\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}$ are the clarified absolute values of observed odds values. In *Table 5*, by substituting the calculated membership degrees of the estimated posibilistic odds values for each cow in (exp $\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]$)quation 23;

$$\mathrm{MDM} = \frac{1}{90}\sum_{i=1}^{90} 0.80 + 1.0 + \cdots + 0.76 + 0.65 \tag{23}$$

Average membership degree value was calculated as MDM = 0.896. It is seen that this calculated value is greater than the value of the tolerance coefficient (h) of $\exp\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right] \geq h = 0.5$, which means that the assumptions required for valid and reliable results are met [22,27]. At the same time, this calculated value states that the fuzzy logistic regression model in equation 16 is a good model for the analyzed data set. According to this test criterion, the average milk yield values in a lactation period such as milk yield of cows in lactation (X_1), weight (X_2), breeds (X_3), age in lactation (X_4), number of milkings per day (X_5) and milking season (X_6) shows that it can be explained by independent variables with a ratio value of 0.896.

Step 10. Another criterion used to evaluate the goodness of fit of the model is the Mean of Squares Errors (MSE). In order to calculate the MSE value, the estimated fuzzy output values calculated in Table 5 were converted to exact values by clarifying the center of gravity with defuzzification $\mathrm{def\,CoG}\left\{\exp\left[\widetilde{W}_j\left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]\right\}$ clarification method in Equation (13).

Thus, the distances between the probability values estimated by clarification and the observed values were calculated. When we want to calculate the $\mathrm{defCoG}\{\exp[\widetilde{W}_i]\}$ value for cow no 1;

$$\mathrm{defCoG}\{\exp[\widetilde{W}_i]\} = \frac{\int_{\exp(-1.98-5.38)}^{\exp(-1.98)} x\left(1-\frac{-1.98-\ln(x)}{5.38}\right)dx + \int_{\exp(-1.98)}^{\exp(-1.98+5.38)} x\left(1-\frac{\ln(x)-(-1.98)}{5.38}\right)dx}{\int_{\exp(-1.98-5.38)}^{\exp(-1.98)} x\left(1-\frac{-1.98-\ln(x)}{5.38}\right)dx + \int_{\exp(-1.98)}^{\exp(-1.98+5.38)} x\left(1-\frac{\ln(x)-(-1.98)}{5.38}\right)dx} = 0.0086 \tag{24}$$

The values calculated as this are substituted in equation (13), it is calculated as: $\mathrm{defCoG}\{\exp[\widetilde{W}_j]\} = \frac{0.0086+41.7754}{-0.1112+27.3669} = 1.533$. The $\mathrm{defCoG}\{\exp[\widetilde{W}_i]\}$ values in *Table 6* were obtained by calculating the clarified (certain) values of the output values for each other cow in this way.

Table 6. The distance between the predicted possibilistic odds values and the observed values by clarification

No	$\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}$	$\mathbf{defCoG}\{\exp[\widetilde{W}_j]\}$	$\left[\mathbf{defCoG}\{\exp[\widetilde{W}_j]\} - \left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]$
1	0.395	1.533	1.138
2	0.166	5.137	4.971
.	.	.	.
89	1.404	3.270	1.866
90	0.030	0.022	-0.008

From each defCoG value calculated in *Table 6*, the observed likelihood (Possibilistic odds) value was subtracted and squared $\left[\mathrm{defCoG}\{\exp[\widetilde{W}_j]\} - \left(\frac{\widetilde{\mu}_i}{[1-\widetilde{\mu}_i]}\right)\right]^2$. Each squared value was placed in the equation (25) and the MSE value was calculated;

$$\mathrm{MSE} = \frac{1}{90}\sum_{i=1}^{90}[1.533-0.395]^2 + [5.137-0.166]^2 + \cdots + [0.022-0.030]^2 \tag{25}$$

The MSE value calculated by Equation (25) was obtained as 4.871 and $\sqrt{\mathrm{MSE}} = 2.207$. This calculated value is the test criterion value that shows the average distance of the fuzzy values estimated with the fuzzy logistic regression analysis model in Equation (16) from the real observation values.

Discussion

Classical logistic regression analysis method is used to model situations where the values of the dependent variable are categorical. The method can generally be used in educational sciences, social studies, and research related to the diagnosis

and treatment of diseases [8,28,29]. However, the use of the method depends on some assumptions. In these studies, reasons such as the expressions of human thoughts and the data sets obtained related to the diagnosis of yield values and animal diseases in livestock, the history of the disease, the unknown environmental factors in the formation of the diseases, and the uncertainty in the symptoms of the disease may cause uncertainties in the diagnostic processes. These assumptions cannot be fulfilled due to the uncertainties arising due to the aforementioned reasons. In these cases, the application of classical statistical methods is not correct [15]. It is a more valid and reliable choice for researchers to use the fuzzy logistic regression analysis method, which is a combination of fuzzy sets and classical statistical theories. It is also a method used in modeling the natural uncertainties in the observation values of the dependent variable or in the relationships. Possibilistic logistic regression analysis method, which is created with the fuzzy logic approach used in different scientific fields, has almost no applications in animal breding in the literature. The application for the estimation of milk yield values of animals belonging to different breeds was discussed for the first time in this study. For fuzzy situations, in the parameter estimations of the fuzzy logistics model, it is thought that the Diamond's Possibilistic method may be a more correct choice to apply to the data obtained for the estimation of yield values in livestock. Doing these and similar studies will gain importance in the solution of all kinds of uncertainties regarding animal husbandry. In the field of veterinary medicine; fuzzy methods were used in various subjects such as estrus detection [30], evaluation of raw milk quality [31], disease diagnosis and determination of risk factors [32]. In their method, fuzzy relation between crisp inputs-crisp output observations is modeled by the proposed model and then compared to the results with a fuzzy neural network method. For some possibilistic methods, one can see the studies done by Taheri and Mirzaei Yeganeh [10], Pourahmad et al.[15], and Atalik and Senturk [8]. Besides, some recent works on distance-based fuzzy logistic regression models are presented by Pourahmad et al.[23], Namdari et al.[28], Salmani et al.[16], and Gao and Lu [17]. Mustafa et al.[9] proposed the fuzzy least square method (FLSM) to determine fuzzy parameters, in that analogue of the conventional normal equations are derived with a suitable metric. Fuzzy logistic regression analysis approach that are presented in these studies are different from other works in the assumptions and optimization method. Pourahmad et al.[15] and Gao and Lu [17] introduced certain least squared fuzzy logistic regression model and evaluated their proposed models by using a capability index for goodness of fit of the model. Namdari et al.[28] used least absolute deviation method to estimate coefficients of a fuzzy logistic regression model and applied measure of performance based on fuzzy distance and a sensitivity index to evaluate the proposed model. Some discussions have been presented offering some modifications on the solution of the above-mentioned exponential possibility regression problems, especially on determining the center of the possibility distribution [3].

To our knowledge, this is the first study of subject "animal husbandry", therefore it can not be compared or discussed with other studies on possibilistic logistic regression in the literature.

In conclusion, it is thought that the approach can be used widely in a short time in studies on animal husbandry and this study can be the basis for similar studies in the future. It can be suggested that researchers who work on this field should consider the possibilistic logistic regression where there is an uncertainity in the dependent variable or in the relationships. It can be concluded that the model can provide the businesses on milk production an efficient and accurate prediction results with minimum deviation by MDM and MSE indices.

Acknowledgements

This research did not receive any specific grant from funding.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 559-566, 2021
DOI: 10.9775/kvfd.2021.25390

RESEARCH ARTICLE

The Effect of *Tarantula cubensis* Extract on Gentamicin-Induced Acute Kidney Injury in Ovariectomized Rats

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Article ID: KVFD-2021-25390 Received: 06.06.2021 Accepted: 18.09.2021 Published Online: 18.09.2021

Abstract

This study examined the effect of *Tarantula cubensis* extract (TCE) on gentamicin-induced acute kidney injury in ovariectomized rats. A total of 40 female Wistar albino rats were randomly divided equally into five groups: Control (C), ovariectomy (O), *Tarantula cubensis* (OT), gentamicin (OG), and gentamicin + *Tarantula cubensis* (OGT). All rats except those in the C were ovariectomized. Kidney damage was created with gentamicin for OG and OGT. The OT and OGT were treated with a single dose of TCE. Blood, sera, and kidney tissue were taken at necropsy for evaluation. Total leukocyte count was higher in the OT compared to the others (P=0.002). Significant increases were also determined in serum urea, creatinine, aspartate aminotransferase, and total protein levels in the OT, OG, and OGT compared to the C and O. The glutathione level was low in the serum and kidney tissue of the OG, and the malondialdehyde level was high compared to the others (P<0.05). As a result of the use of TCE in gentamicin-induced acute kidney injury in ovariectomized rats, serum creatinine, urea, and malondialdehyde levels decreased in the OGT compared to the OG, the glutathione level increased, and the severity of histopathological findings decreased to milder levels. As a result; single dose of TCE partially reduced kidney damage in rats with gentamicin-induced acute kidney injury.

Keywords: *Acute kidney injury, Gentamicin, Rat, Tarantula cubensis*

Ovariektomili Ratlarda Gentamisin İle İndüklenen Akut Böbrek Hasarına *Tarantula cubensis* Ekstraktının Etkisi

Öz

Bu çalışmada ovariektomili ratlarda gentamisinle indüklenen akut böbrek hasarına *Tarantula cubensis* ekstraktının (TCE) etkisinin araştırılması amaçlandı. Çalışmada Wistar albino, dişi, yetişkin, 40 rat kullanıldı. Randomize şekilde her grupta 8 rat olacak şekilde 5 gruba ayrıldı. Gruplar; Kontrol grubu (K), ovariektomi grubu (O), *Tarantula cubensis* grubu (OT), gentamisin grubu (OG), gentamisin + *Tarantula cubensis* grubu (OGT) olarak belirlendi. K dışındaki tüm ratlara overektomi operasyonu yapıldı. OG ve OGT'de gentamisin ile böbrek hasarı oluşturuldu. OT ve OGT'de tek doz TCE ile tedavi uygulandı. Değerlendirme için ötenazi sonrası kan, serum ve böbrek dokusu alındı. Total lökosit sayısı OT'de diğer gruplara oranla yüksek bulundu (P=0.002). Serum biyokimyasal parametrelerde, K ve O'ya kıyasla OT, OG ve OGT'de serum üre, kreatinin, aspartat aminotransferaz ve total protein seviyelerinde önemli artışlar belirlendi. OG'de serum ve böbrek dokusunda glutatyon seviyesi düşük, malondialdehit seviyesi diğer gruplara kıyasla yüksek bulundu (P<0.05). Ovariektomili ratlarda gentamisinle indüklenen akut böbrek hasarında TCE kullanımı sonucunda, OGT'de, OG'ye kıyasla serum kreatinin, üre ve malondialdehit seviyesinin düştüğü, glutatyon seviyesinin yükseldiği ve histopatolojik bulguların şiddetinin daha hafif düzeylere indiği görüldü. Sonuç olarak gentamisinle indüklenen akut böbrek hasarında TCE tek doz uygulanması böbrekteki hasarı kısmen azalttı.

Anahtar sözcükler: *Akut böbrek hasarı, Gentamisin, Rat, Tarantula cubensis*

INTRODUCTION

Antibiotics are classified as killing bacteria or preventing their growth. Aminoglycosides act by killing bacteria. Antibiotics exert their negative effects on bacteria in different ways. Aminoglycosides show their effect on bacteria by inhibiting protein synthesis. This narrow-spectrum antibiotic group is especially effective against

How to cite this article?

Akyüz E, Makav M, Kuru M, Bayram P: The effect of *Tarantula cubensis* extract on gentamicin-induced acute kidney injury in ovariectomized rats. *Kafkas Univ Vet Fak Derg,* 27 (5): 559-566, 2021.
DOI: 10.9775/kvfd.2021.25390

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Gram-negative bacteria. Although useful in many diseases, these drugs are also associated with some adverse side effects, including ototoxic, nephrotoxic, neurotoxic, and teratogenic effects. Even at normal treatment doses, they can cause kidney function losses when used for more than five days. Because of such side effects, antibiotics of the aminoglycoside group should not be the primary treatment choice. Especially in patients with renal dysfunction, they should be used with caution [1]. Medications such as aminoglycoside antibiotics, anti-inflammatory drugs, and those used for neoplastic conditions can have toxic effects on organs such as the kidneys [2-4]. These drugs can cause renal damage at high rates, even when used at standard treatment doses. Therapeutic doses of gentamicin may reportedly lead to acute renal failure in approximately 20% of patients [5-7]. The cause of kidney damage due to gentamicin has not been fully elucidated; however, some theories have been postulated. One such theory supported with experimental studies asserts that the negative effects of free oxygen radicals may play a role [8-10]. Different doses and application methods have been used to induce kidney damage in rats in experimental studies [9,10]. Gentamicin, which can cause loss of kidney function even with normal doses, is known to produce rapid effects at high doses. Nephrotoxicity has been induced by administering gentamicin intraperitoneally at a dose of 100 mg/kg/day for eight days [10].

Homeopathy originated as a form of treatment based on the principle that like things are treated with like, meaning that while something can cause disease symptoms when used in high doses, it can treat the same disease with minimal doses [11,12]. Supportive treatment is needed in some diseases in the field of veterinary medicine. One such application is *Tarantula cubensis* extract (TCE), a homeopathic product obtained from *T. cubensis* spiders used as supportive therapy [13]. Primarily used in veterinary medicine, it is prescribed to relieve edema, treat traumatic-necrotic disorders, infectious diseases, rapid epithelization, and some types of cancer [14,15]. Its effectiveness in different diseases in various animal breeds has been reported [13-17]. TCE is certified as a homeopathic product in veterinary medicine and available for use in some target species. Due to its antiphlogistic and regenerative properties, it is used in inflammatory diseases, ulcers and purulent lesions [13].

Glutathione in tissues occurs as a result of the peroxidation of fatty acids. It is intended to protect against oxidative damage, and the intracellular concentrations are very high. It was reported that the resistance of the proximal tubule to oxidative damage compared to the medullary parts is due to high glutathione levels [17,18].

Malondialdehyde is widely used in the measurement of oxidative stress. It can be detected in the blood as well as measured in the urine. It correlates well with the degree of lipid peroxidation since there is no specific indicator for the oxidation of fatty acids [17].

The kidneys are under the influence of sex hormones and factors. Estrogens have a positive role in the progression of some kidney diseases. Estrogen has more regenerative and immune system enhancing effects. In addition, estrogens have nephroprotective effects [19]. It has been reported that sex hormones have important effects on kidney damage [20,21]. During the cycle in rats, changes will occur in estrogen, progesterone and some hormone levels. Therefore, rats were ovariectomized to more accurately assess the effect of TCE on gentamicin-induced kidney damage and to minimize the effects of sex hormones.

This study aimed to examine the effects of TCE on gentamicin-induced kidney injury in ovariectomized rats by evaluating glutathione and malondialdehyde levels as well as hematological and histopatological parameters.

Material and Methods

Ethical Statement

Upon the approval of the Local Ethics Committee of Animal Experiments of Kafkas University (KAU-HADYEK/2020-100), this study was conducted in the Department of Internal Medicine of the Faculty of Veterinary Medicine, Kars, Turkey.

Animals

Wistar albino rats were obtained from Kafkas University Experimental Animals Application and Research Center, Kars, Turkey. Daily rat pellet feed and water consumption of rats kept in standard cages were tracked. During the study, all rats were provided with a relative humidity of 40%-60%, optimal room temperature (22ºC), and 12 h of light and 12 h of darkness. A total of 40 female rats weighing 255-300 g were utilized for the study. Following a 15-day adaptation period, the rats were randomly divided into five groups: Control (C), ovariectomy (O), *Tarantula cubensis* (OT), gentamicin (OG), and gentamicin + *Tarantula cubensis* (OGT), each having eight rats.

Methods

Ovariectomy procedures were performed on all rats, except for those in the control group (C). The rats were anesthetized with a combination of 5-10 mg/kg/IP xylazine HCl (Rompun®2%-Bayer) and 35-50 mg/kg/IP ketamine HCl (Ketalar®-Pfizer) for the operation. The median line area was shaved and cleaned, and the skin, muscle layers, and peritoneum were incised to reach the ovaries. The right and left ovaries and suspensory ligaments and vessels were ligated with 3-0 polyglactin 910 (Vicryl® Ethicon) and removed. The peritoneum and muscles were closed with simple continuous sutures and the skin with simple interrupted sutures. Enrofloxacin (Baytril 10%®, Bayer, Germany) was injected intramuscularly at a dose of 10 mg/kg for four days postoperatively. The incision areas of the rats were checked daily for peritonitis and inflammation [22].

Once the rats were determined to be healthy 15 days after the operation, kidney damage (acute tubular necrosis) was created with gentamicin, and TCE (Theranekron D6®, Richter Pharma, Austria) was applied to the experimental groups.

Group (C) did not undergo an ovariectomy or any other application. All rats in the other four groups were ovariectomized, and group (O) did not receive any additional application. Group (OT) received a single dose (0.3 mg/kg/SC) of TCE. Group (OG) was administered gentamicin (80 mg/kg/IP) once a day for one week to induce acute kidney injury [23]. Group (OGT) was administered gentamicin (80 mg/kg/IP) for one week and a single dose of TCE (0.3 mg/kg/SC).

Blood and Tissue Samples Taken

All rats were euthanized (cervical dislocation) under the general anesthesia at the end of the study, and blood samples were taken from the heart into serum tubes with gel (BD Vacutainer®, BD, UK) and tubes with K_2EDTA (BD Vacutainer®, BD, UK). Blood samples taken for serum were kept at room temperature for about one hour and centrifuged at 3000 rpm for ten min (Hettich Rotina 380R®, Hettich, Germany). Kidney tissue samples taken for biochemical analysis were homogenized in phosphate buffer, and homogenates were removed. All samples were stored at -20ºC until analysis.

Biochemical and Hematological Analyses

Blood samples in K_2EDTA were assessed for total leukocyte count (WBC x10^3/µL) and other hematological parameters using a complete blood count device (VG-MS4e®, Melet Schloesing, France). Serum urea, creatinine, total protein (TP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured with a fully automatic biochemistry device (Mindray BS120®, Mindray Medical Technology Istanbul, Turkey). Measurement of malondialdehyde from serum and tissue homogenates was carried out according to the Yoshioka et al.[24] procedure and glutathione measurement according to the Beutler et al.[25] procedure. A spectrophotometric microplate reader device (Spectramax Plus®, Marshall Scientific, Product Code: MD-SMP, USA) was used to obtain the data.

Histopathological Procedure

Histopathological analyses were performed in the Histology and Embryology Laboratories, Faculty of Medicine, Kafkas University, Turkey. The kidney tissues were fixed with 10% buffered formalin for 72 h, washed under running water for 15-20 min to remove formalin, and then dehydrated by passing through graded alcohol (75%, 96%, 100%). After clearing the tissue with xylene and paraffin infiltration, paraffin blocks were prepared. Sections of 5-µm thickness were stained with hematoxylin-eosin (Sigma-Aldrich®, Merck, Germany). Micrographs were taken using a light microscope with a DP21 camera system (Olympus BX43®, Japan) to evaluate morphological alterations. Sections were graded according to histopathological findings as none (-), mild (+), moderate (++), or severe (+++).

Immunohistochemical Analysis

After deparaffinization with xylene and rehydration with graded alcohols (100%, 96%, 75%) for two min, sections were boiled with 10mM citrate buffer for ten min in a microwave for antigen retrieval. The sections were lined with a hydrophobic pen and washed with 0.1M phosphate buffer sodium (PBS). To block the endogenous peroxidase activity, they were incubated with 3% hydrogen peroxidase solution for five min at room temperature (RT), washed with PBS, and incubated with blocking solution for ten min at RT. After removing the blocking solution, the sections were incubated with anti-BAX (FNab000810, 1:100 dilution) and anti-Caspase9 (FNab01295, 1:100 dilution) primary antibody at +4ºC overnight. The kidney sections were washed with PBS and incubated with secondary antibodies for ten min at RT. Prior to horseradish peroxidase (HRP) polymer incubation for ten min at RT, blocks were washed with PBS. The sections were incubated with 3,3-diaminobenzidine (DAB) solution for two min, and the DAP reaction was stopped with distilled water. Harris hematoxylin was used as a counterstain. Sections were washed with distilled water and dehydrated with graded alcohols (75%, 96%, 100%) for two min and mounted with Entellan®-A Thermo Scientific™ UltraVision™ Quanto Detection System HRP DAB kit (Thermo Scientific™ TL-060-QHD) was used for hydrogen peroxide, blocking solution, secondary antibody, and HRP polymer.

For immunohistochemical analysis, five different areas from each slide were photographed under 20× magnifications. H-SCORE rates were calculated with the H-SCORE=Σ Pi (i+1) formula [micrographs staining densities (i), pixel ratios (Pi)] [26].

Statistical Analyses

Data were given as mean ± standard error of mean (SEM). The groups were not in accordance with the normal distribution according to the histogram, Q-Q graph method and Shapiro-Wilk test. The Kruskal-Wallis H test was used for multiple comparisons of the groups, and the Mann-Whitney U test was used for pairwise comparisons. Adjusted P values were taken into account by applying Bonferroni correction to the P value obtained after the Mann-Whitney U test. SPSS (SPSS Version 23.0®, Chicago, IL, USA) program was used for all statistical analyses. The differences between the groups in terms of the parameters examined were considered significant at the $P<0.05$ level.

Results

Hematological and Biochemical Evaluation

The total leukocyte count was higher in the OT group compared to the other groups (P=0.002). Regarding other

hematological parameters, statistical significance was found between the groups in terms of lymphocytes, granulocytes, monocytes, erythrocytes, hematocrit percentage, hemoglobin, and platelet counts (P<0.05) *(Table 1)*. Regarding serum biochemical parameters, significant increases were determined in serum urea, creatinine, aspartate aminotransferase, and TP levels in the OT, OG, and OGT groups compared to the C and O groups (P<0.05) *(Table 2)*. The OG group had the lowest glutathione level in serum and kidney tissue and the highest malondialdehyde level (P<0.05) *(Table 2)*.

Histopathological Evaluation

Hematoxylin-eosin staining was performed to evaluate the morphological changes in kidney tissues caused by TCE in rats with nephrotoxicity induced by gentamicin *(Fig. 1)*. Kidney tissues of group C had a normal appearance, and group O was similar to group C. The glomerulus and Bowman's space were normal in the OT group; however, different from group O, mild glomerular segmentation was observed. Swelling and cytoplasmic vacuoles were observed in the proximal tubule cells in the OG group, with

Table 1. *Hematological data in the study according to groups*

Parameters	Groups (Mean±SEM)					P Value
	C	O	OT	OG	OGT	
Total leukocytes count (x10³/µL)	5.52±0.35^{a}	4.87±0.71^{a}	10.27±1.16^{b}	5.87±0.74^{ab}	6.88±0.53^{ab}	0.002
Lymphocytes count (x10³/µL)	5.20±0.31	4.20±0.63	7.68±1.06	3.99±0.70	4.97±0.65	>0.05
Monocytes count (x10³/µL)	0.13±0.02^{a}	0.17±0.04^{a}	0.66±0.09^{b}	0.48±0.05^{b}	0.49±0.05^{b}	<0.001
Granulocytes count (x10³/µL)	0.19±0.04^{a}	0.51±0.13^{ac}	1.93±0.06^{b}	1.39±0.06^{bc}	1.42±0.22^{bc}	<0.001
Red blood cell count (x10⁶/µL)	7.11±0.18^{a}	7.29±0.24^{a}	12.58±2.34^{b}	7.17±0.11^{a}	7.52±0.13^{a}	0.038
Mean red cell volume (fL)	70.91±0.66^{a}	71.55±0.92^{a}	66.60±1.25^{ab}	64.21±0.55^{b}	62.60±1.21^{b}	<0.001
Hematocrit (%)	50.361.24^{a}	52.03±1.49^{a}	70.85±8.78^{b}	45.94±0.88^{a}	46.86±0.90^{a}	0.004
Mean erythrocyte hemoglobin (pg)	23.04±0.30^{a}	23.93±0.38^{ab}	19.38±2.03^{a}	24.69±0.18^{b}	23.41±0.20^{ab}	0.011
Mean hemoglobin volume (g/dL)	32.51±0.16^{a}	33.53±0.67^{a}	31.88±2.31^{a}	38.51±0.11^{b}	37.55±0.67^{b}	<0.001
Erythrocyte distribution width (fL)	10.45±0.44^{ab}	11.73±0.17^{a}	9.68±0.25^{b}	9.89±0.17^{b}	9.55±0.26^{b}	0.001
Hemoglobin (g/dL)	16.40±0.40^{a}	17.43±0.37^{a}	21.30±1.48^{b}	17.71±0.36^{a}	17.59±0.24^{a}	0.001
Platelet count (x10³/µL)	1049.88±27.29	2268.75±483.80	4477.00±1378.70	872.13±76.90	1097.38±114.47	>0.05
Mean platelet volume (fL)	5.16±0.04	5.23±0.13	5.30±0.06	5.21±0.05	5.15±0.04	>0.05
Platelets (%)	0.54±0.02^{ab}	1.14±0.23^{ab}	2.42±0.75^{a}	0.45±0.04^{b}	0.56±0.06^{ab}	0.017
Platelet distribution width (fL)	7.20±0.17^{a}	7.23±0.23^{a}	7.95±0.23^{ab}	8.55±0.23^{b}	8.21±0.09^{b}	0.007

C: *Control group,* ***O:*** *Ovariectomized group,* ***OT:*** *Tarantula cubensis group,* ***OG:*** *Gentamicin group,* ***OGT:*** *Gentamicin + Tarantula cubensis group,* ***SEM:*** *Standard error of mean*
$^{a\text{-}c}$ *The mean values with different letters in the same line represent the difference between groups. P<0.05: Expresses statistical significance*

Table 2. *Biochemical parameters in serum and kidney tissue according to groups*

Parameters	Groups (Mean±SEM)					P Value
	C	O	OT	OG	OGT	
Alanine aminotransferase (U/L)	51.11±4.02	47.13±3.33	50.51±5.53	62.03±5.80	54.74±2.40	>0.05
Aspartate aminotransferase (U/L)	134.62±8.54^{a}	137.03±6.81^{a}	141.49±5.37^{a}	177.84±15.64^{b}	195.51±6.73^{b}	<0.001
Urea (mg/dL)	56.57±5.98^{a}	57.36±7.06^{a}	68.74±3.23^{ab}	94.38±16.02^{b}	79.56±7.62^{ab}	0.017
Creatinine (mg/dL)	0.66±0.11^{a}	0.84±0.09^{a}	0.93±0.06^{a}	2.53±0.08^{b}	2.25±0.23^{b}	<0.001
Total protein (g/dL)	7.06±0.19^{a}	7.15±0.13^{ab}	7.70±0.15^{bc}	8.08±0.19^{c}	7.73±0.11^{bc}	<0.001
Glutathione (umol/mL)	1.42±0.11^{ab}	1.31±0.09^{ab}	1.52±0.12^{b}	1.09±0.05^{a}	1.12±0.06^{a}	0.007
Malondialdehyde (nmol/mL)	1.43±0.06^{a}	1.46±0.05^{a}	1.51±0.14^{a}	2.15±0.11^{b}	2.02±0.12^{b}	<0.001
Kidney tissue glutathione (umol/g)	7.90±0.12^{a}	8.15±0.26^{a}	9.33±0.50^{a}	7.62±0.25^{a}	11.89±0.81^{b}	<0.001
Kidney tissue malondialdehyde (nmol/g)	9.51±0.34^{a}	9.69±0.49^{a}	10.58±0.40^{ab}	13.51±0.56^{c}	12.25±0.45^{bc}	<0.001

C: *Control group,* ***O:*** *Ovariectomize group,* ***OT:*** *Tarantula cubensis group,* ***OG:*** *Gentamicin group,* ***OGT:*** *Gentamicin + Tarantula cubensis group.* ***SEM:*** *Standard error of mean.* $^{a\text{-}c}$ *The mean values with different letters in the same line represent the difference between groups. P<0.05: Expresses statistical significance*

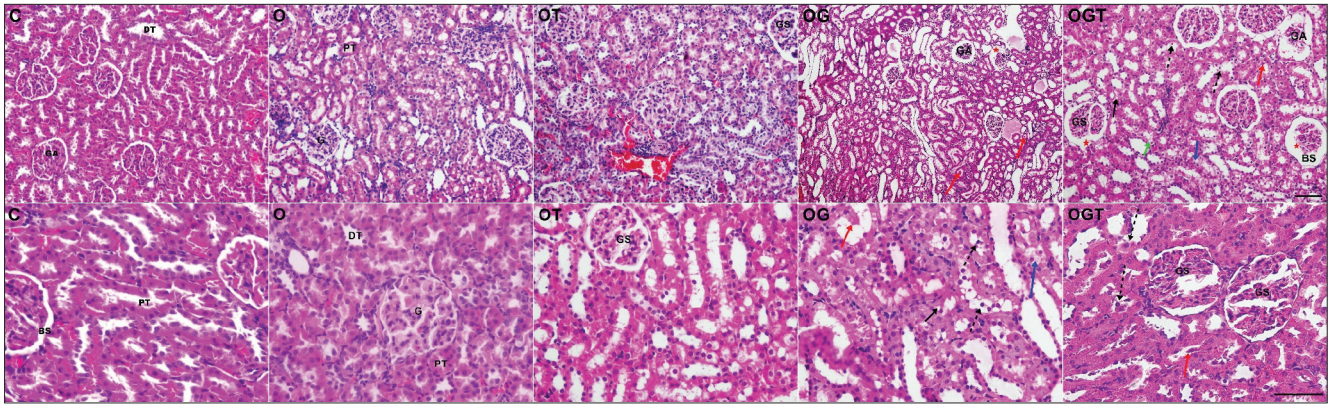


Fig 1. Photomicrographs showing the histopathological changes of hematoxylin-eosin stained rat kidney (H&E 20X and 40X). Kidney tissues of group C and group O had a normal appearance and the OT group had mild glomerular segmentation (GS). In the OG and OGT groups, it was observed cytoplasmic vacuoles *(black arrow),* glomerular atrophy (GA), the dilatation of Bowman's space *(star),* hyaline cylinders *(red arrow)* in tubule lumen, cytoplasmic degeneration *(blue arrow),* cell in proximal tubule lumen *(black dashed arrow)* and enlarged cells *(green arrow);* although there was variation in their density. G: Glomeruli, DT: Distal tubule, PT: Proximal tubule, C: Control group, O: Ovariectomized group, OT: *Tarantula cubensis* group, OG: Gentamicin group, OGT: Gentamicin + *Tarantula cubensis* group. Bar (top row): H&E X20, Bar (bottom row): H&E X40

Table 3. *Score of histological damage in the kidney tissue*

Parameters	**C**	**O**	**OG**	**OT**	**OGT**
Dilatation in Bowman space	-	-	+++	-	++
Glomerular segmenting	-	-	+++	+	+++
Cells in tubules lumen	-	-	++	-	++
Cytoplasmic vacuole in tubules cells	-	-	+++	+	++
Hyaline cylinders in tubules lumen	-	-	++	-	+

Sections were evaluated according to histopathological findings as none (-), mild (+), moderate (++) and severe (+++). ***C:*** *Control group,* ***O:*** *Ovariectomized group,* ***OT:*** *Tarantula cubensis group,* ***OG:*** *Gentamicin group,* ***OGT:*** *Gentamicin + Tarantula cubensis group*

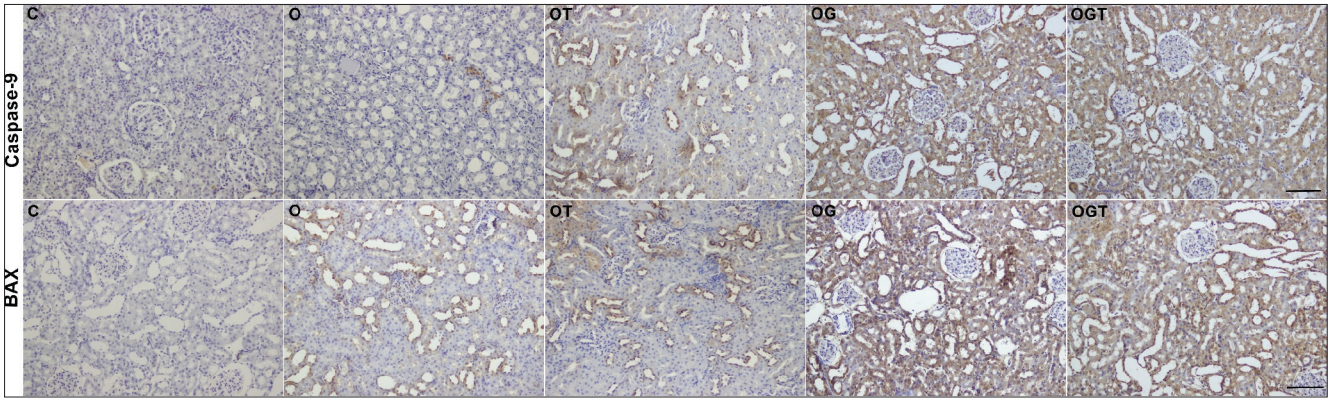


Fig 2. Photomicrographs showing immunohistochemical labeling with Bax and Caspase9 primary antibody in rat kidney (20X magnification). Brown staining indicates positive immune-reactivity. Caspase-9 and Bax proteins are secreted when cells are prone to apoptosis. Positive immune-reactivity areas indicate the susceptibility of cells to apoptosis. Compared to the other groups, the OG and OGT groups have more apoptotic cells rates because of positive immune-reactivity. C: Control group, O: Ovariectomized group, OT: Tarantula cubensis group, OG: Gentamicin group, OGT: Gentamicin + Tarantula cubensis group

some areas showing cytoplasm degeneration. Compared to groups C and O, dilatation in Bowman's space, atrophy of glomeruli, and segmentation stood out. The OG group exhibited many cells in the tubule lumen, indicative of acute tubular necrosis. In addition, the hyaline cylinder increase in the proximal tubule lumen was remarkable. Comparison of the OGT group and OG group showed dilatation in Bowman's space, cells in the tubule lumen indicative of acute tubular necrosis, and the proportion of hyaline cylinders in the proximal tubule lumen were decreased. Glomerular segmentation was at the same level. Score of histological damage in the kidney tissue give in *Table 3*. It was observed that apoptosis was induced as a result of nephrotoxicity caused by gentamicin in the OG

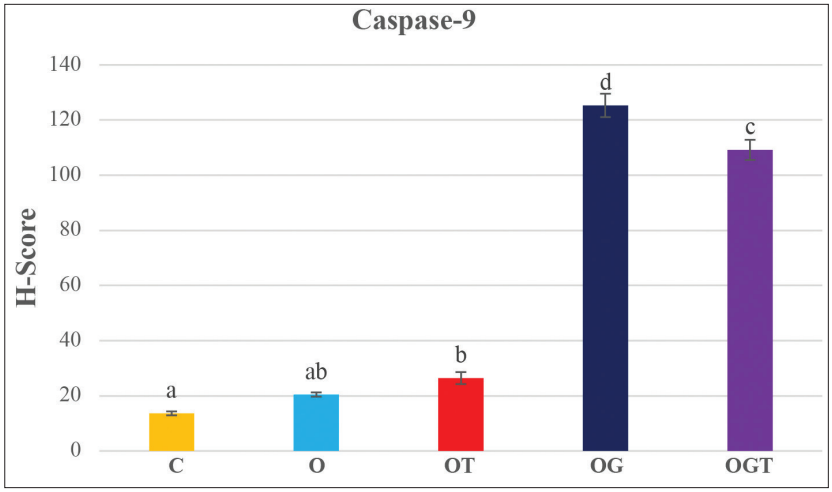
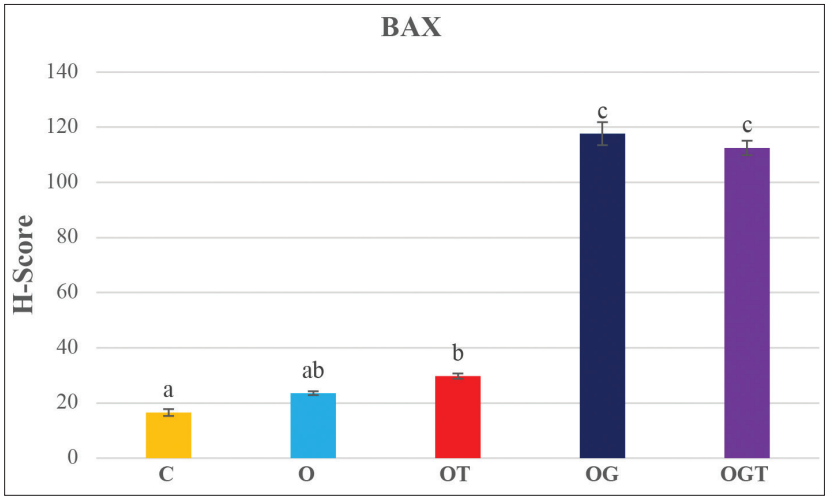


Fig 3. H-score of Caspase-9 by groups. **C:** Control group, **O:** Ovariectomized group, **OT:** *Tarantula cubensis* group, **OG:** Gentamicin group, **OGT:** Gentamicin + *Tarantula cubensis* group. [a-c] Different letters represent the difference between groups. P<0.05: Expresses statistical significance

Fig 4. H-score of BAX by groups. **C:** Control group, **O:** Ovariectomized group, **OT:** *Tarantula cubensis* group, **OG:** Gentamicin group, **OGT:** Gentamicin + *Tarantula cubensis* group. [a-c] Different letters represent the difference between groups. P<0.05: Expresses statistical significance



and OGT groups *(Fig. 2)*. The H-score of BAX and caspase-9 according to the groups was statistically significant (P<0.05) *(Fig. 3* and *Fig. 4)*.

Discussion

Changes in hematological and serum biochemical parameters provide physicians important information related to the severity of diseases, the effectiveness of treatment, metabolic events, and organ functions [27,28]. A study conducted on rats showed similar results in terms of hematological parameters with our OG group, especially in WBC. We believe that the higher WBC level in the OT group compared to the other groups is due to the regenerative properties of TCE on the tissue.

Gentamicin administered at high doses to induce nephrotoxicity enters the cytoplasm by disrupting the cell membrane. Gentamicin in the cytosol activates the intrinsic apoptosis pathway by affecting mitochondria [29], thus increasing Bax [29,30] expression. Bax, in turn, increases cytochrome-c production, which increases apoptosis. Some previous studies showed that cytochrome-c activates the caspase pathway [31]. The current study showed that gentamicin triggered apoptosis as a result of nephrotoxicity, which is consistent with the literature.

One of the most important side effects of gentamicin is nephrotoxicity. Certain increases in serum creatinine indicate nephrotoxicity, which in general, is directly proportional to the dose and duration of administration [32,33]. A study conducted on rats found increased serum urea and creatinine levels in a gentamicin group compared to a control group [34]. Similar results in the OG and OGT groups in the current study show that nephrotoxicity occurred. Another study determined that TCE had a protective effect on nephrotoxicity and decreased urea and creatinine concentrations [17]. Consistent with the literature, the current study determined that serum creatinine and urea concentrations were lower in the OGT and OG groups, which showed that TCE partially reduced acute kidney injury. We reason this result is due to the antiphlogistic and regenerative properties of TCE as well as its curative effect on necrotic tissues. A study conducted on rats reported that the malondialdehyde level in a gentamicin group was higher than in control and TCE groups [17]. Glutathione in tissues is intended to protect against oxidative damage, and malondialdehyde is widely used to

measure oxidative stress [17,18,35]. Our results suggested that an increased oxidative stress load led to low glutathione and high malondialdehyde levels in the OG group, which is also in line with the literature. Histopathological changes in the kidney tissues of rats with gentamicin-induced nephrotoxicity were also in line with the results of previous studies [34,36]. Similar to the literature, glomerular atrophy, hyaline cylinders in tubule lumens [37], tubular vacuolization, cell desquamation in tubule lumens [38], and glomerular segmentation [39] were observed in the OG group. With the addition of TCE, it was observed that Bowman's space dilatation, vacuoles in the tubular cell cytoplasm, and the proportion of hyaline cylinders in the tubule lumen decreased. However, cell desquamation and the rate of glomerular segmentation in the tubule lumen did not change. Histopathological comparison of the OGT and OG groups showed only slightly different results, which is thought to be due to the fact that only a single dose of TCE was injected.

Based on our results, we determined that the single-dose administration of TCE provided a partial protective and therapeutic effect. We believe that the protective and therapeutic effect of TCE will be enhanced when administered repeatedly. Additional studies are needed to confirm this assumption.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Funding Support

There is no funding source.

Conflict of Interest

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

Author Contributions

EA, MM and MK conceived and supervised the study. EA and MK collected and analyzed data. EA and MM made laboratory measurements. PB applied the histopathological examination of the study. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 567-573, 2021
DOI: 10.9775/kvfd.2021.25867

RESEARCH ARTICLE

Butyric Acid Bacteria Culture Solution Improves Hyperglycemia in Alloxan-Induced Diabetes Mellitus Rats

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Article ID: KVFD-2021-25867 Received: 08.04.2021 Accepted: 31.08.2021 Published Online: 10.09.2021

Abstract

Butyric acid bacteria (BAB) are the primary intestinal flora present in all mammalian digestive tracts. Prior studies have found the association between decreased intestinal BAB population and development of diabetes, and BAB was suggested as a new treatment of type 2 diabetes. However, few studies have examined the effect of BAB on type 1 diabetes (DM1), which is frequently diagnosed in pet animals. Therefore, the aim of this study was to examine the therapeutic effects of BAB culture solution in the DM1 model. Thirty female rats were included for induction of DM1 by alloxan (200 mg/kg, IP). After one week, DM1 was developed in 13 rats (blood glucose level >300 mg/dL) which were then treated with BAB culture solution at a dose rate of 300 µL/kg/day for two weeks. The result revealed a reduction in blood glucose level ($P<0.05$) and improvement of polydipsia and polyuria in six diabetic rats; meanwhile, 7 rats did not respond to the treatment. The blood pressure showed no change. In conclusion, the administration of BAB culture solution alleviates symptoms of DM1 by improving glycemic control in the model. The effectiveness of BAB as an alternative or supportive therapy for the treatment of DM1 needs further studies on pet animals.

Keywords: *Alloxan, Butyric acid bacteria, Hyperglycemia, Rat diabetes model*

Bütirik Asit Bakteri Kültür Solüsyonu Alloksan İle Diabetes Mellitus Oluşturulmuş Ratlarda Hiperglisemiyi İyileştirir

Öz

Bütirik asit bakterileri (BAB), tüm memeli sindirim sistemlerinde bulunan birincil bağırsak florası etkenleridir. Önceki çalışmalar, azalan bağırsak BAB popülasyonu ile diyabet gelişimi arasındaki ilişkiyi saptamışlar ve BAB, tip 2 diyabetin yeni bir tedavisi olarak önerilmiştir. Bununla birlikte, BAB'nin, evcil hayvanlarda sıklıkla teşhis edilen tip 1 diyabet (DM1) üzerine etkisini inceleyen az sayıda çalışma mevcuttur. Bu nedenle, bu çalışmada, DM1 modelinde BAB kültürü solüsyonunun terapötik etkilerinin incelenmesi amaçlanmıştır. Çalışmada, alloksan (200 mg/kg, IP) ile DM1 oluşturulması için otuz dişi rat kullanılmıştır. Alloksan uygulamasından bir hafta sonra 13 ratta DM1 oluşturulmuş (kan şekeri seviyesi >300 mg/dL) ve sonraki iki hafta boyunca bu ratlar 300 µL/kg/gün dozu ile BAB kültür solüsyonu ile tedavi edilmiştir. Tedavi sonrası, diyabetik 6 ratta kan glukoz seviyesinde bir azalma ($P<0.05$) ve polidipsi ve poliüri tablolarında iyileşme saptanırken, 7 rat tedaviye yanıt vermemiştir. Kan basıncında bir değişiklik görülmemiştir. Sonuç olarak, BAB kültür solüsyonunun uygulanması, modeldeki glisemik kontrolü iyileştirerek DM1 semptomlarını hafifletmektedir. DM1 tedavisinde alternatif veya destekleyici bir terapi olarak BAB'nin etkinliği, evcil hayvanlarda yapılacak daha fazla çalışmaya ihtiyaç duymaktadır.

Anahtar sözcükler: *Alloksan, Butirik asit bakterileri, Hiperglisemi, Rat diyabet modeli*

How to cite this article?

Shimada K, Hara S, Goya S, Mandour AS, Kitpipatkun P, Hamabe L, Uemura A, Takizawa J, Tanaka R: Butyric acid bacteria culture solution improves hyperglycemia in alloxan-induced diabetes mellitus rats. *Kafkas Univ Vet Fak Derg,* 27 (5): 567-573, 2021.
DOI: 10.9775/kvfd.2021.25867

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Introduction

Populations of butyrate-producing bacteria, such as *Clostridium butyricum*, a gram-positive spore bacillus, are recognized as important intestinal bacteria which possess great health benefits [1,2]. Because of the spore forming characteristic of *C. butyricum*, it can be utilized when administrated orally for therapeutic applications as it can pass through stomach to the intestine without degradation by the gastric acid [3]. Various studies have demonstrated the treatment benefits of BAB. In this regard, *C. butyricum* administration normalizes intestinal flora [4] and inhibits the activity of some pathogenic bacteria [5,6]. In humans, commercialized products containing BAB are available as nutritional supplements, which tout probiotic effects [7]. In the dairy industry, butyrate-producing bacteria are administered to livestock to improve intestinal healing and enhance appetite [8,9]. In broiler chickens, butyrate products are known to improve levels of hepatic enzymes and uric acid [10].

Recently, BAB culture solution, unlike the existing *C. butyricum* products, containing various contents such as BABs, produced-butyric acid and BAB culturing environment, have been developed which is expected to have the potential to normalize the intestinal environment after pancreatic disorder. The BAB fermentation was effective in suppressing the growth of pathogenic microorganisms, such as *E. coli*, *S. aureus* (ATCC25923), *C. difficile* (ITO) and *C. sporogenes* (GAI95048) [5,6]. After culturing, vitamins (B2, B6, B12 and C) and amino acids (Alanine, Valine, β-Alanine and γ-Aminobutyric acid) were produced with BAB fermentation (non published data). Administration of BAB culture solution was also effective in the treatment of atopic dermatitis and was suggested to decrease mast cell population and mitigation of allergic symptom in mice models (non published data).

Diabetes mellitus (DM) is a common ailment in small animal veterinary medicine, and the only available treatment modality is glycemic management with insulin. Home monitoring of DM, particularly measuring blood glucose, is often difficult for small patients such as hamsters and ferrets, so dietary treatments play a major role in management of diabetic complications. Recently, intestinal flora, including butyric-producing bacteria, was reported to improve lifestyle diseases such as metabolic disorders and obesity in human patients [4,11-13]. A moderate degree of gut bacterial disorder has been reported in type 2 DM, in which the usefulness of butyrate-producing bacteria in metabolism is decreased concomitantly with increasing number of opportunistic pathogens [11,12]. In a study of obese mice, *C. butyricum* inhibited progression of metabolic syndrome to type 2 DM and further improved insulin resistance by reducing cytokine production characteristic of diabetes onset [14]. Furthermore, *C. butyricum* alleviated hyperglycemia in type 2 DM mice [15]. Notably, previous studies identified the BAB therapeutic approach as a new treatment modality for type 2 DM through in depth studies of the anti-inflammatory and antioxidant effects of *C. butyricum* and its associated butyric acid [15]. Most of the studies of BAB for DM used spontaneous animal model and were focused on suppressing the onset of hyperglycemia [1]. Until now, no prior study highlighting the efficacy of BAB culture solution in alleviating the hyperglycemia in type 1 DM, an irreversible pancreatic disorder, in a small animal clinical veterinary setting has been reported. Therefore, the aim of the present study is to evaluate whether oral administration of BAB culture solution controls hyperglycemic state in alloxan (ALX)-induced DM1 rat model.

Material and Methods

Ethical Statement

This study was conducted with pre-approval from the ethical committee to use experimental animals in Tokyo University of Agriculture and Technology (approval number: 29-74).

Animals

Three-months-old, 30-Sprague-Dawley female rats (CHARLES RIVER LABORATORIES JAPAN, INC., Japan) were bred in-house. The average body weight of 30 rats was 379.31 g. All rats were cared under the same management conditions. Animals were single-housed, identified based on cage number, and maintained under *ad libitum* feeding and a 12 h light/dark cycle. The used diet was supplied by a commercial company (Oriental yeast CO., LTD., Tokyo, Japan) which provides energy and protein levels of 359 kcal/100g and 23.1 g/100g, respectively.

Induction of DM and Administration

Figure 1 shows the schema of the present study. DM was induced by alloxan injection (ALX; Alloxan Monohydrate, Tokyo Chemical Industry CO., LTD., Japan; 200 mg/kg IP, dissolved in saline) [16]. One week after injection, rats exceeding blood glucose level of 300 mg/dL were used as diabetic rats for subsequent experiments [17] and were started with the administration of BAB culture solution.

Administration of Butyric Acid Bacteria Culture Solution for Treatment of DM1

Butyric acid bacteria culture solution (ACE BIO PRODUCT CO., Japan) was administered by mixing with the drinking water for an estimated dosage of 300 μL/kg/day for two weeks. When this dosage did not significantly affect the blood glucose level, the dosage was increased to double and then to triple the initial dose for the preceding two weeks. The dosage of BAB was considered effective when blood glucose level became <200 mg/dL and the experiment was terminated.

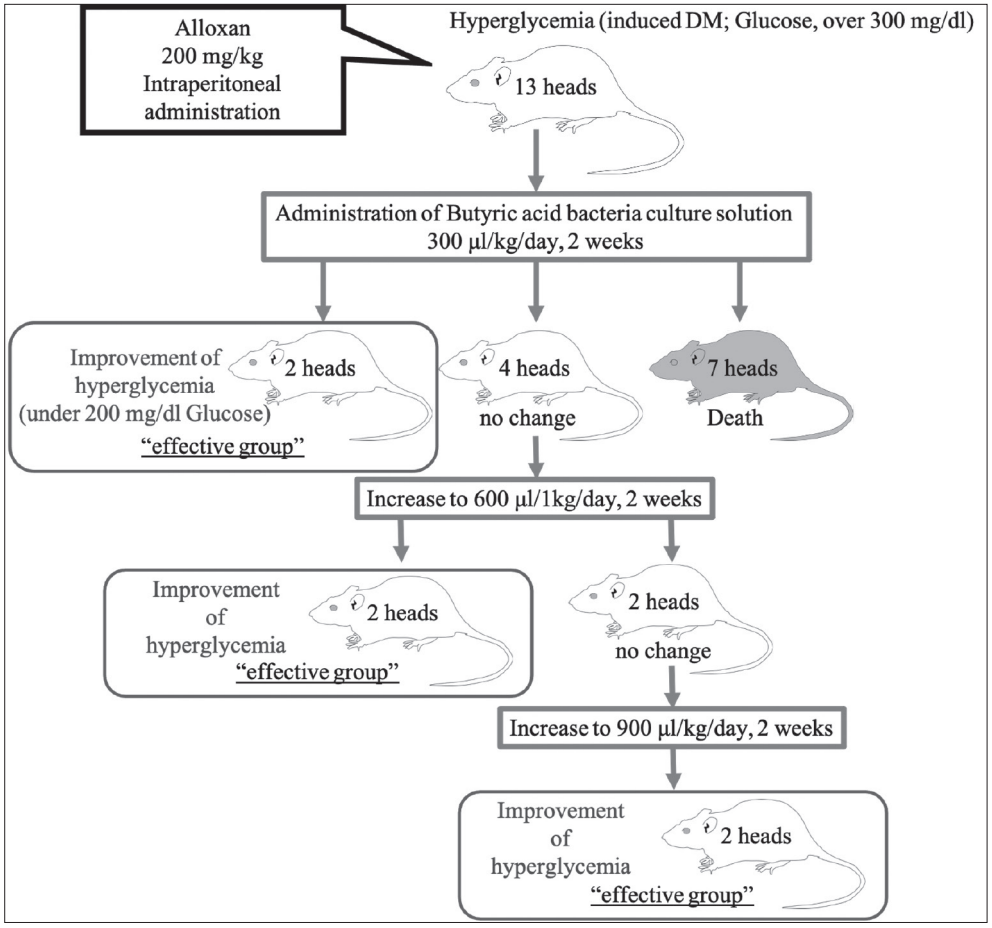

Fig 1. Schematic representation of the present study. The total number of Alloxan-induced DM rats was 13 heads, and started to administrate butyric acid bacteria culture solution from 300 µL/kg/day for 2 weeks. After 2weeks, butyric acid bacteria solution was increased to 600 µL/kg/day, and next 2 weeks were further increased to 900 µL/kg/day. Finally, six rats showed improved hyperglycemia

Blood Sampling and Blood Glucose Monitoring

Blood samples were collected prior to DM induction and every week thereafter. Conscious rats were placed in a holding tool for blood pressure measurement (Rat holder M/L, Muromachi Kikai CO., Ltd., Japan). While the animals were restrained, a small amount of blood was sampled from the tail vein using a 1 mL syringe (TOP Corporation, Japan) and 30G needle (Becton, Dickinson and Company, Japan). For measurement of blood glucose level, blood was dropped onto the blood glucose monitoring system (Glucose PILOT, technicon internal Inc., Japan).

Blood Pressure Measurement

Blood pressure measurement was conducted before and after DM induction and at the end of the experiment. Rats were placed in the holding tool for blood pressure measurement, and measurement was performed on the tail using a rodent blood pressure measurement device (BP MONITOR FOR RATS & MICE Model MK-2000, Muromachi Kikai CO., Ltd., Japan). After the pulse pressure waveform stabilized, data acquisition was conducted five times for each animal. Systolic, diastolic and mean blood pressure were recorded.

Statistical Analysis

Sample size was determined based on the outcomes and calculation performed with the G*Power 3.1.9.2 software (University Kiel, Germany, 1992-2014) [18], assuming a moderate effect of BAB culture solution on DM1 rat model according to Cohen [19] with 0.37 effect size. The data were categorized as before induction of DM (negative control, NC), after induction of DM1 (ALX-DM), and after administration of BAB culture solution (BAB). Data were expressed as mean ± standard division through one-way ANOVA analysis and a $P<0.05$ was considered statistically significant. Each data figure was prepared using software for statistical analysis (GraphPad Prism, version5.0a, GraphPad Software, USA).

Results

Induction of Type 1 DM

A week after the ALX injection, 13 rats out of 30 (43.3%) developed DM1 as indicated by elevation of the blood glucose (BG) level. After ALX injection, the BG was significantly increased from the basal level ($P<0.05$). These

Table 1. *Fluctuation of blood glucose levels of individual animals in the effective group*

Parameter	NC	ALX-DM	BAB
Blood glucose level (mg/dL)	140	318	100
	82	338	126
	149	451	87
	152	390	160
	166	352	153
	131	308	154
Average	136.7	359.5*	130.0**

*Before alloxan-injection is expressed as NC (negative control), and ALX-DM indicates one week after alloxan-injection. BAB means post administration of BAB culture solution. Significant differences are indicated by * (vs. NC, P<0.05) and ** (vs. ALX-DM, P<0.05)*

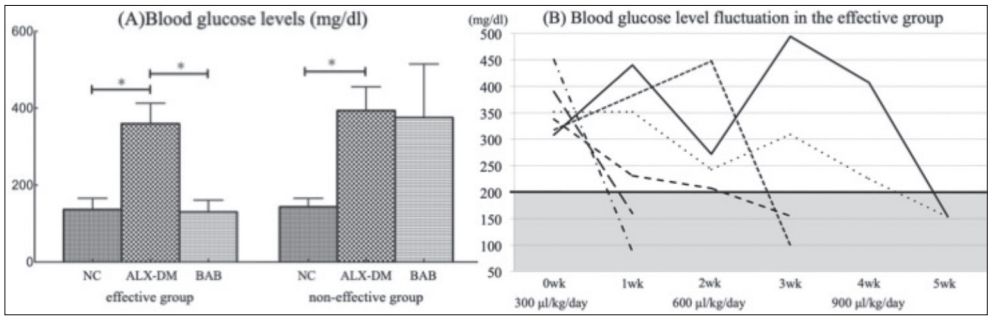

Fig 2. Changes (A) and fluctuation (B) in blood glucose level. Blood glucose level was significantly increased to over 300 mg/dL after ALX injection (ALX-DM). This significant increase of blood glucose level suggested that induction of diabetes mellitus was successful. Blood glucose level after administration of butyric acid bacteria culture solution was significantly decreased (BAB)

rats were ultimately included in the analysis as type 1 DM model.

BAB Alleviates Hyperglycemia in Type 1 DM

The BAB culture solution was administered daily at an initial dose of 300 µL/kg/day followed by duplication of doses in non-responsive rats. *Table 1* shows fluctuations in blood glucose level in the effective group. Among thirteen DM1 rats, only six rats showed significant decrease in blood glucose level after administration of BAB culture solution and were grouped as the "effective group" *(Fig. 2-A)*. However, the effective dose, which is the dose required to reduce the BG to the non-diabetic level, was not the same among the effective group. On the other hand, seven rats did not respond to BAB administration and died. These rats were classified as the "non-effective group".

Notably, in the present study, we used increasing dose of BAB depending on the response of the blood glucose level. These doses were equally suppressing DM1 (2 rats for each dose). In this regard, low dose, 300 µL/kg/day, decreased blood glucose level in two rats, moderate dose (600 µL/kg/day) decreased blood glucose level in two additional rats, and the high dose (900 µL/kg/day) was effective in another two rats *(Fig. 2-B)*.

Before induction of the DM, the blood glucose level was not significantly different between the two groups *(Fig. 3)*.

Blood Pressure Measurement

Blood pressure measurements before and after ALX injection are shown in *Table 2*. The data revealed no significant difference between groups at the basal time and after development of type 1 DM (P>0.05). Additionally, significant differences were not observed between effective and non-effective groups after administration of BAB culture solution.

General Physical Conditions

In the effective group, concomitantly with reduction in blood glucose level, polyuria, as indicated by the cage environment, was improved. Macroscopically the pigmentation of the abdominal hair was reduced. However, urine excretion was not directly quantified. The average body weight of the effective group was 369.5 g.

Discussion

The new trend for treatment of diseases recommends usage of probiotics and prebiotics on large scale as a supportive or replacement therapy, particularly metabolic ones. Natural products are organic, safe, and characterized by multi-pathway mechanisms to combat diseases. Recently, BAB has been discussed as a potential therapeutic target for treatment of metabolic disorders including DM [4,13-15]. The

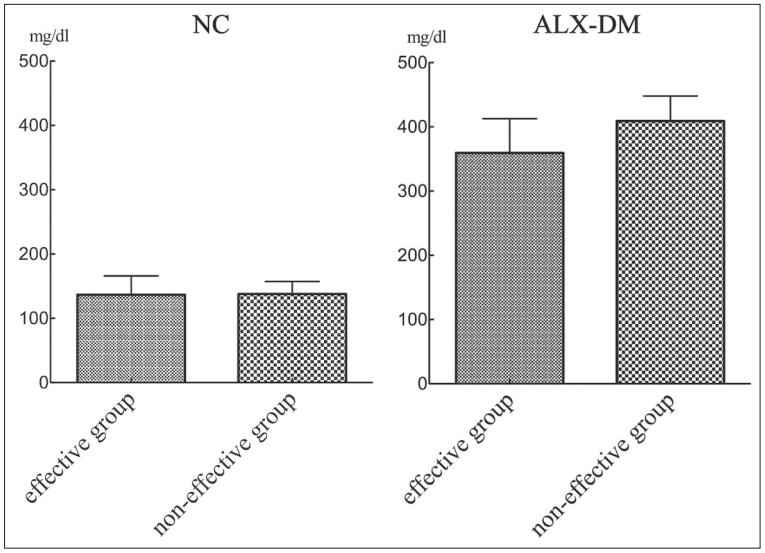

Fig 3. Severity of diabetic mellitus in each group. NC, negative control (before alloxan injection); ALX-DM, alloxan induced diabetes mellitus. The effective group (decreased blood glucose level with butyric acid bacteria culture solution treatment) and the non-effective group had no significant differences in blood glucose level before and after injection of alloxan

Table 2. *Blood pressure before and after alloxan injection*

Groups	Systolic (mmHg)		Mean (mmHg)		Diastolic (mmHg)	
	Pre ALX	Post ALX	Pre ALX	Post ALX	Pre ALX	Post ALX
Effective	94.0±15.3	101.2±22.9	63.8±9.8	70.3±10.0	48.7±11.8	52.7±8.2
Non-effective	102.0±6.3	93.9±12.2	66.4±8.3	64.8±11.9	48.5±11.8	50.3±13.9

Results are expressed as average ± SD. No significant differences were detected

current study investigated the hypoglycemic efficiency of BAB culture solution in DM1 model.

Alloxan induces irreversible destruction of pancreatic islet β cells [20]. It was used in the present study to induce DM1, which resulted in elevation of blood glucose [21]. At least 50% of diabetic dogs have DM1, and epidemiological factors closely match those of the latent autoimmune diabetes of adult form of human DM1 [22]. In the present study, the results suggest that BAB culture solution is effective against DM1. Interestingly, 46% (6 rats) of the enclosed rats showed euglycemia after oral administration of BAB culture solution. After alloxan administration, the blood glucose level which exceeded 300 mg/dL was definitely decreased to non-diabetic level after BAB administration, which may be attributed to the ability of BAB to attenuate pancreatic inflammation [4]. BAB, especially *C. butyricum*, was reported to decrease pancreatic damage through reducing inflammation cytokine level [15]. From the present study, this BAB culture solution was suggested to relieve pancreatic inflammation. On the other hand, the remaining rats (54%) did not respond to BAB administration, and resulted in hyperglycemia. This difference in outcomes may be due in part to differences in the extent of pancreatic disorder; however, the present study did not perform histopathology and was unable to confirm the level of pancreatic disorder. Because there was no statistical difference in the blood glucose level before BAB culture solution administration between these two groups, blood glucose alone may not be sufficient for evaluation of DM severity. In the present study, the dosage of BAB culture solution was sequentially increased until an effective dose for glycemic control was reached. This effect was observed immediately after the increase in BAB culture solution dosage. Accordingly, it can be said that the effectiveness of BAB against type 1 DM is likely to be dose-dependent. In aquatic animals, *C. butyricum* was shown to exert a dose-dependent effect on the immune system as probiotics, and have been administered up to 1.0×10^{12} CFU/kg [23]. In the present study, BAB culture solution contained 1.0×10^{5} CFU/mL of *C. butyricum*, and administration dose was decided from previous mice experiment conducted by the manufacturer of BAB culture solution. Moreover, *C. butyricum* has no known contraindications and considered as a potential complementary therapy for effective control of DM [14]. Accordingly, administration of BAB culture solution in "non-effect" rats of this study failed to acclimatize the hyperglycemia state even at final dose, because the dose of administration may not be appropriate for the alloxan-induced DM model.

In human medicine, DM is a well-known risk factor for cardiovascular disease [24] including cardiomyopathy and vascular endothelial disorders leading to hypertension that sometimes exacerbate type 1 and type 2 DM, and these interrelationships have been widely investigated [25-29]. In a previous study, anti-hyperglycemic compounds were effective for cardiovascular disorders associated with DM [30]. In the present study, the effect of BAB culture solution

on cardiac complications associated with diabetes was evaluated by measuring blood pressure. However, BAB culture solution did not significantly affect blood pressure. Because this diabetic model was acute [31], the evaluation may have been conducted prior to development of cardiovascular complications. In a previous study, alloxan-DM rats were donated for the cardiovascular examination 8 weeks after induction [32]. Therefore, longer term observation will be necessary to fully assess the therapeutic effects of BAB culture solution on the cardiovascular system in DM.

In small-sized animals such as hamsters, hedgehogs, and birds, therapeutic modalities for diabetes are focused on preventing death and severe complications and improving hygiene control [33], rather than on curative therapy. Osmotic diuresis secondary to hyperglycemia worsens cage conditions in DM animals due to polydipsia and polyuria [34]. In the present study, the cage environment was notably improved after administration of BAB culture solution. We speculate that this change was due to improvement in polydipsia and polyuria, a common clinical sign of DM. This finding suggested that BAB administration increases the hygienic condition of the cage, which in turn may prevent secondary infections or complications in DM. Although quantitative assessment was not conducted, decreased consumption of drinking water and reduction of hair pigmentation due to polyuria were noticeable. Improving the living environment will significantly improve the quality of life for DM animals that receive BAB culture solution. The results of the present study indicated that BAB culture solution for dietary control of DM in small animals is convenient and safe for small animal caretakers and had multiple beneficial outcomes, including glycemic control, improved quality of life, and decreased water consumption and subsequently improved polyuria. Although the mechanism of action for BAB culture solution is not fully understood, some previous studies suggested that this modality improves insulin resistance.

This is a preliminary study which highlights the utility of BAB culture solution in DM1. However, further studies addressing the dose response manner, histopathological and molecular pathways are warranted.

In conclusion, BAB culture solution is an important potential complementary therapeutic for type 1 DM, and the utility of this modality is particularly notable for small animal veterinary medicine, including exotic animals.

Acknowledgment

Authors thank Kotomi Sato, Taisei Doko and Kenjiro Shiraishi for supporting this research to take care of diabetes rats.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

K. Shimada planned the present study, conducted this experiment, and wrote this manuscript. S. Hara and S. Goya conducted and supported this experiment. A. S. Mandour, P. Kitpipatkun and A. Uemura supported to write this manuscript. L. Hamabe corrected English of this manuscript. J. Takizawa researched about butyric acid products. R. Tanaka supported and supervised the present study.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 575-581, 2021
DOI: 10.9775/kvfd.2021.25880

RESEARCH ARTICLE

The Effect of Dragon Fruit Extract on Experimental Mesentery Arterial Ischemia-Reperfusion in Rats

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Article ID: KVFD-2021-25880 Received: 09.04.2021 Accepted: 02.08.2021 Published Online: 06.08.2021

Abstract

We hypothesized that dragon fruit extract (DFE) might be a protective agent in oxidative stress damage that develops against ischemia and reperfusion injury in the rat intestine. The rats used in the study were randomly divided into 8 groups, with 6 animals in each group. The HEALTHY group was not induced by ischemia and not given DFE (Group 1). The DFE1000 group was not induced by ischemia; however, DFE was given 1000 mg/kg (Group 2). Ischemia was induced for 1 h in groups 3, 4, 5, 6, 7 and 8. The clamps were then removed to allow reperfusion for 45 min (Groups 6, 7 and 8). DFE was given to rats at doses of 500mg/kg and 1000 mg/kg 30 min before Ischemia (I) and Ischemia/Reperfusion (I/R) administration. At the end of the experiment, histopathological, biochemical and molecular analyses were performed on the intestinal tissues. While glutathione peroxidase, superoxide dismutase, glutathione levels increased significantly in the I+DFE500, I+DFE1000, I/R+DFE500 and I/R+DFE1000 groups compared to the I and I/R groups, there was a significant decrease in tumor necrosis factor-α, Caspase 3 and malondialdehyde ($P<0.05$). In addition, the loss of tissue integrity, and the increase of inflammatory cells were decreased in the treatment groups. As a result, it was determined that DFE has a strong protective role against oxidative damage in the treatment of intestinal ischemia-reperfusion injury.

Keywords: *Dragon fruit extract, Intestine, Ischemia/reperfusion, Oxidative stress, Rat*

Ejder Meyvesi Ekstraktının Sıçanlarda Deneysel Mezenter Arteriyel İskemi-Reperfüzyon Üzerine Etkisi

Öz

Ejder meyvesi ekstraktının (EME), sıçan bağırsağında iskemi ve reperfüzyon hasarına karşı gelişen oksidatif stres hasarında koruyucu bir ajan olabileceğini varsaydık. Çalışmada kullanılan sıçanlar her grupta 6 hayvan olacak şekilde rastgele 8 gruba ayrıldı. SAĞLIKLI grubu, iskemiyle indüklenmedi ve EME verilmedi (Grup 1). EME1000 grubu, iskemiyle indüklenmedi; ancak EME 1000 mg/kg verildi (Grup 2). Grup 3, 4, 5, 6, 7 ve 8'de iskemi 1 saat süreyle indüklendi. Daha sonra 45 dakika reperfüzyona izin vermek için klempler çıkarıldı (Grup 6, 7 ve 8). Sıçanlara İskemi (I) ve İskemi/Reperfüzyon (I/R) uygulamasından 30 dakika önce 500 mg/kg ve 1000 mg/kg dozlarda EME verildi. Deney sonunda bağırsak dokularında histopatolojik, biyokimyasal ve moleküler analizler yapıldı. Glutatyon peroksidaz, süperoksit dismutaz, glutatyon seviyeleri I + EME500, I + EME1000, I/R + EME500 ve I/R + EME1000 gruplarında I ve I/R gruplarına göre önemli ölçüde artarken, tümör nekroz faktörü-α, Kaspaz 3 ve malondialdehit seviyelerinde anlamlı azalma oldu ($P<0.05$). Ayrıca tedavi gruplarında doku bütünlüğü kaybı ve inflamatuar hücre yoğunluğu azaldı. Sonuç olarak, bağırsak iskemi-reperfüzyon hasarının tedavisinde DFE'nin oksidatif hasara karşı güçlü bir koruyucu rolü olduğu belirlendi.

Anahtar sözcükler: *Ejder meyvesi ekstraktı, Bağırsak, İskemi/reperfüzyon, Oksidatif stres, Sıçan*

How to cite this article?

Sahin L, Toktay E, Yayla M, Kilicle P, Harmankaya A, Ozic C, Yigit S, Aydemir Celep N: The effect of dragon fruit extract on experimental mesentery arterial ischemia-reperfusion in rats. *Kafkas Univ Vet Fak Derg,* 27 (5): 575-581, 2021.
DOI: 10.9775/kvfd.2021.25880

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Introduction

Ischemia (I) is the reduced or interrupted blood flow to a tissue and reperfusion is the state of restoring blood flow and oxygen to ischemic tissue [1]. Damage caused by ischemia can be exacerbated by reperfusion [2] and the gastrointestinal tract tissues are highly susceptible to Ischemia/Reperfusion (I/R) damage in the body [3]; intestines are the mostly affected organs [4]. I/R may also cause damage following abdominal aortic aneurysm, small bowel transplant, strangulated hernia, hypovolemic and septic shock [5]. Acute mesenteric ischemia (AMI) occurs with the sudden interruption of the blood supply of the small intestines and as a result, cellular damage and necrosis have been observed. Diarrhea, malabsorption, short bowel syndrome, and death may occur as a result of intestinal I/R damage [6]. The severity of ischemia in the intestine depends on the duration of the ischemia, the level of mesenteric artery occlusion, and the rate of collateral flow [5].

Intestinal ischemiaca damage can lead to the rapid progression of tissue necrosis, accelerating the production of reactive oxygen, as well as the rapid translocation of intestinal bacteria to the mucosa. In addition, the release of cytokines, such as inflammation precursor interleukin 1β and tumor necrosis factor-α, may lead to cell damage, severe metabolic disorders, and multi-organ failure due to inflammation [1]. AMI, is a serious life-threatening surgical problem [7]. The mortality rate in patients that are misdiagnosed, or late diagnosis is 60 to 80% [5] and irreversible damage occurs after the first 6 h. Although blood flow can be restored with urgent surgical intervention, it should be managed in light of the high oxidative damage that occurs. From this point of view, foods containing high antioxidants can attenuate oxidative damage by collecting superoxide radicals emitted. Natural products such as astragalus membranaceus, ostiole, green tea, and *Nigella sativa* with high antioxidant content, have been shown to cause antioxidative effects on the intestinal mucosa [8-10].

The red dragon fruit *(Hylocereus polyrhizus)* has anti-inflammatory, antiradical and high antioxidant capacities. In recent studies, red dragon fruit extract is preferred due to its antimicrobial, antioxidant, anticancer, hypo-cholesterolemic, and prebiotic effects [11-13]. The fruit has also scavenging property against alkyl and hydroxyl radicals. Because of this feature, it contributes to the balancing of oxidative stress [14]. Moreover, this fruit is rich in organic acids, protein and other minerals such as potassium, magnesium, calcium, and vitamins [15,16].

The experimental intestinal I/R model is one of the important models used to reduce oxidative stress caused by reactive oxygen species, especially during reperfusion in the small intestine.

Our study suggests that dragon fruit extract (DFE) may have a protective role in the treatment of acute mesenteric artery ischemia and reperfusion. Our aim in this study was to investigate if the dragon fruit can be used in the treatment of damage resulting from small intestine mesenteric artery ischemia. Accordingly, we analyzed the capacity of the extract to eliminate reactive oxygen species (ROS) in the experimental mesenteric artery I/ R model.

Materials and Methods

Ethics Statement

The Local Ethics Committee of Animal Experiments of Kafkas University approved this study (Approval No: KAÜ-HADYEK: 2020/046).

Extraction

Dragon fruits were obtained from the Mersin/Erdemli, Dragon Fruit Greenhouse. The peel of the fruit was removed and the parts eaten as fruit were sliced. They were dried sparsely on blotter paper in an environment free from direct sunlight and provided with dry airflow. The dried fruit slices were ground in a grinder. The ground fruits were weighed with 0.1 mg sensitivity and approximately 50 g were taken into the cartridge washed with extraction solvent. The cartridge was placed in 500 mL Soxhlet extractor and 650 mL of solvent was added to the boiling flask. The solvent was extracted (9-12 siphons) until it was clear. 96% ethanol was used as the extraction solvent and after the obtained extract was filtered through the blue band filter paper and its particles were removed, the solvents were evaporated at 40-50°C with a rotary evaporator in the balloons brought to constant weight.

Animals

Forty-eight Sprague-Dawley female rats with an average weight of 200-250 g and 10-12 weeks old were obtained from the Kafkas University Experimental Research and Application Center. The rats were given enough *(ad libitum)* water and pellet feed during the experiment. The animals were housed at optimal room temperature and humidity levels.

Rats were randomly divided into 8 groups with 6 animals in each group *(Table 1)*.

Study Design

The animals were anesthetized through the intraperitoneal (i.p.) administration of a combination of 15 mg/kg xylazine HCl (Rompun®-Bayer-Turkey) and 100 mg/kg ketamine (Ketalar®-Pfizer-Turkey). A 4 cm incision was made in the ventral midline approach at the level of the umbilicus of the rats. Then the jejunum part of the small intestine was taken out. The bloodstream from the superior mesenteric artery was stopped for 45 min, with the help of vascular

Table 1. *Experimantal model and groups*

Groups	Animal Numbers	Groups Abbreviation	Group Descriptions
Group 1	n:6	HEALTHY	Only laparotomy was performed in the Healthy group
Group 2	n:6	DFE1000	Only laparotomy was performed and DFE 1000 mg/kg was given
Group 3	n:6	I	45 min of ischemia period was applied with laparotomy
Group 4	n:6	I+DFE500	Rats were given DFE 500 mg/kg orally by gavage 30 min before ischemia, followed by a 45 min ischemia period
Group 5	n:6	I+DFE1000	The rats were given DFE 1000 mg/kg orally by gavage 30 min before ischemia, followed by a 45 min ischemia period
Group 6	n:6	I/R	45 min of ischemia followed by a 1 h reperfusion period were applied to the rats by laparotomy
Group 7	n:6	I/R+DFE500	After 45 min of ischemia, 1 h reperfusion period was applied. DFE 500 mg/kg was given orally by gavage to rats before 30 min of reperfusion
Group 8	n:6	I/R+DFE1000	After 45 min of ischemia, 1 h reperfusion period was applied. DFE 1000 mg/kg was given orally by gavage to rats before 30 min of reperfusion

clamps. After initiation of ischemia, the intestines were reinserted into the abdomen and the incision area was closed with sutures. The ischemia-only group (Group 3) was euthanized after 45 min of ischemia. In the ischemia and reperfusion groups, the abdominal incision was reopened and the clamps were removed at the end of 45 min of ischemia. They were exposed to reperfusion injury by providing bloodstream again. These groups (Group 6, 7, and 8) were euthanized 1 h after the start of reperfusion (The intestines were then reinserted into the abdomen and sutured closed) [17,18].

At the end of the study, animals were euthanized by high dose thiopental sodium (i.p.) under anesthesia in compliance with ethical principles. The jejunum segment of the intestine was observed and the intestinal organs were grasped with pens. Collected tissues were stored at 3.7% formaldehyde for histopathological and at -80°C for biochemical examination.

Extract Application Procedure

While DFE was given orally by gavage 30 minutes before ischemia to I+DFE500 (Group 4) and I+DFE1000 (Group 5) groups; I/R+DFE500 (Group 7) and I/R+DFE1000 (Group 8) groups were given 30 min before reperfusion. Experimental groups and applied procedures are given in *Table 1*.

Histolologic Analyses

Intestine tissues were rapidly fixed in 3.7% solution formaldehyde for 48 h. After fixation, all samples for histological tissue processing were routinely performed. According to histological tissue processing, all tissues were passed through increasing alcohol (50, 60, 70, 80, 96, 99-1 h) series. Subsequently, 3 series of xylene were kept in solution (3x15 min). Finally, it was kept in molten soft and hard liquid paraffin. At the end of the follow-up procedure, tissues were individually blocked in paraffin. After tissue processing, 5 µm thick sections were taken from each paraffin block for histopathological examination. Intestine tissue slides were stained with Mallory's trichrome stain.

Biochemical Analyses

For the determination of oxidant/antioxidant parameters [Malondialdehyde (MDA), Glutathione (GSH)], 100 mg of tissue was weighed for each animal. All tissues were homogenized in a homogenizer device. For biochemical studies, while MDA levels in supernatants were measured colorimetrically according to the methods reported by Yoshioko et al.[19] at 535 nm, GSH levels are measured colorimetrically as regards Beutler et al.[20] at 412 nm by invitrogen elisa reader.

Molecular Analysis

- RNA isolation and cDNA synthesis

In the study, Ribonucleic acid (RNA) isolation was first made from tissues. This isolation was performed with TRIzole (Sigma).

- Total RNA isolation with TRI solution (Tri Reagent)

Tissues were dissected using the freeze-thawing method and centrifuged at 7000 rpm for 15 min, then turned into tissue pellets. The supernatant was removed, 750 mL TRI was added onto the pellet, and homogenate was obtained. The homogenate was left at room temperature for 10 min and 200 µL chloroform was later added to the homogenate for each 1 mL of TRI. The samples were tightly capped and shaken vigorously for 20 sec, then the shaken mixture was left at room temperature for 10 min. It was centrifuged at 12.000 g for 15 min at 4°C. The supernatant was then carefully removed and transferred to a new tube. 0.5 mL of isopropanol was added to the upper phase separated for each mL of TRI used in the 4th step. The samples obtained were kept at room temperature for 15 min and samples were then centrifuged at 4°C, 8 min, and 12.000 g. The supernatant was removed, and the RNA pellet was

washed by adding at least 1 mL of 75% ethanol per ml of TRI used in step 4th and vortexing. The washed sample was centrifuged at 4°C for 5 min at 7500 g. Ethanol was removed and the pellet was dried in a fume hood for 15-20 min. RNA was resolved with 80 µL ddH_2O (Sterile ultra-pure water) and the RNAs thus obtained were measured using the spectrophotometric method.

- cDNA Synthesis

cDNA was obtained using the Fermentas Revert Aid First Strand cDNA Synthesis Kit (#1622), each sample containing 5 µg of RNA. All steps are done according to the kit procedure. First, the RNA sample obtained from RNA isolation was taken into ice in 0.5 mm PCR tubes so that it would react with 5 g, and 1 µL of oligo dT18 was added and the final volume was completed to 12 µL with sterile distilled water. The reaction tube was kept at 70°C for 5 min and then left on the ice. To the reaction tube in ice, respectively were added 4 µL 5x Reaction buffer, 1 µL Ribolock Ribonuclease inhibitor, and 2 µL 10 mM dNTP mix, then incubated at 37°C for 5 min. At the end of the incubation, 1 µL of M-MuLV reverse transcriptase enzyme was added. The tube containing the prepared mixture was first kept at 42°C for 60 min. At the end of the incubation, the reaction tube was incubated at 70°C for 10 min to inhibit the enzyme. At the end of this time, the reaction tube was placed on ice and subsequently kept at 20°C for use in studies.

- Expression Analysis by Reverse Transcription Polymerase Chain Reaction

Rat primer sequences of RT-PCR are detailed in *Table 2*. It was aimed to determine the expression levels of Glutathione Peroxidase (GPX), Superoxide Dismutase (SOD), Tumor Necrosis Factor- alpha (TNF-α) and Caspase-3 (Cas-3).

- Generation of cDNA Copy

PCR reaction 2.5 µL 10X buffer, 2.5 µL 25 mM $MgCl_2$, 2 µL 2.5 µM dNTP mix, 2.5 µL F, 2.5 µL R, 0.5 µL cDNA template (1:10 dilution), 0.2 µL Taq DNA Polymerase enzyme (5 U/µL) 12.3 µL ddH_2O was added, so that the final volume was 25 µL. The PCR program used: 5 min at 94°C, 50 sec at 94°C, 1 min at 50-60°C, 50 sec at 72°C, 5 min at 72°C. The material was stored in the device at 4°C until it was taken.

Statistical and Semi-quantitative Analysis

The data of our study were statistically evaluated with the IBM 20.00 SPSS software program. The groups were compared to Tukey's post-hoch tests from the one- way ANOVA multiple comparison test with a value of $P<0.05$ considered statistically significant.

Results

Histopathological Findings

In the Healthy group *(Fig. 1-A)* and the DFE1000 group *(Fig. 1-B)*, healthy-looking villus structures were observed in the small intestine mucosa. No pathological condition was found in the epithelium and lamina propria. In the I group, loss of tissue integrity of the villi structures in the small intestine mucosa and inflammatory cells and edema in the lamina propria were observed. However, areas of hemorrhage were seen in the submucosa and locally in the mucosa *(Fig. 1-C)*. In the I +DFE500 *(Fig.*

Table 2. *Rat Primer sequences of RT-PCR*

Gene	Primer	Annealing
GPX	F: CAGTTCGGACATCAGGAGAAT R: AGAGCGGGTGAGCCTTCT	60
SOD	F: ATGTGTCCATTGAAGATCGTGTGA R: GCTTCCAGCATTTCCAGTCTTTGTA	60
TNF-α	F: ACTGAACTTCGGGGTGATTG R: GCTTGGTGGTTTGCTACGAC	60
Cas-3	F: CATTTCTTTAGTGATAAAA R: ATCATGGGATCTGTTTCTTT	60

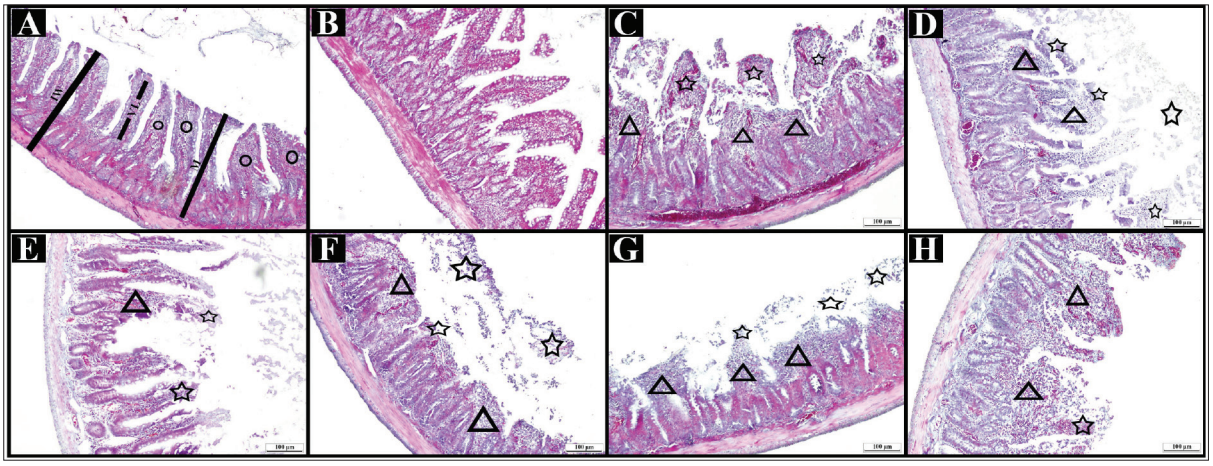

Fig 1. Triple staining findings in intestinal tissue. IW: Intestinal wall, VL: Villus, M: Mucosa, Round: Lamina propria, Triangle: Inflammatory area, Star: Loss integrity of tissue. Magnification: x20

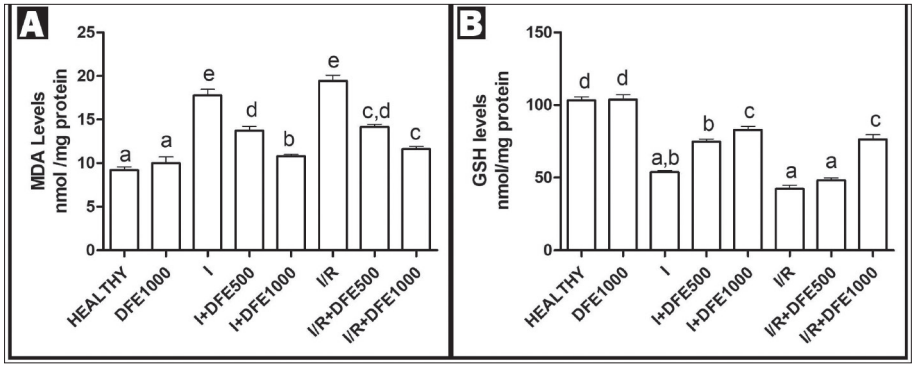

Fig 2. Comparison of biochemical MDA and GSH between groups (I: Ischemia, I/R: Ischemia and reperfusion, DFE: Dragon Fruit Extract)

Fig 3. Comparison of molecular GPX, SOD, TNF-α and Caspase-3 between groups (I: Ischemia, I/R: Ischemia and reperfusion, DFE: Dragon fruit extract)

1-D) and in the I +DFE1000 *(Fig. 1-E)* groups, we observed that loss of tissue integrity of the villi structures in the small intestine mucosa occurred mostly in the superficial region. However, inflammatory cells and edema were observed in the superficial mucosa. In these groups, areas of hemorrhage were decreased compared to the ischemia group.

As seen in the I group, the villi with loss of tissue integrity in the I/R group were completely detached from their basal parts and a naked mucosa image was observed. Inflammatory cells and edema were seen *(Fig. 1-F)*. In the I/R+DFE500 group, the villi were relatively protected compared to I/R group, but a naked mucosa image was also present in this group. Similarly, inflammatory cells and edema were seen *(Fig. 1-G)*. In the I/R+DFE1000 group, compared to thel/R group, the villi were clearly preserved for epithelial losses, but a bare mucosa image was also present. Inflammatory cells and edema were decreased compared to the I/R and the I groups *(Fig. 1-H)*.

Biochemical Findings

MDA levels in the I and I/R groups were higher than those in the healthy group. These levels were significantly reduced in a dose-dependent manner in the ischemia and I/R+DFE treatment groups *(Fig. 2-A)* ($P<0.05$). We determined that the GSH level reduced after ischemia. However, it increased in the treatment groups. GSH levels were especially low in the I/R groups and increased in a dose-dependent manner in the DFE treatment groups *(Fig. 2-B)* ($P<0.05$).

Molecular Findings

In the healthy and DFE1000 groups, the expression levels of GPX, SOD, TNF-α, and Cas-3 were determined to be at the same level as Glyceraldehyde 3-phosphate dehydrogenase used as the control gene. In the ischemia and I/R groups, GPX, SOD expression levels decreased, while TNF-α and Cas-3 levels increased compared to the healthy group. While the levels of GPX, SOD increased in the I and I/R treatment groups compared to the I and I/R groups, TNF-α and Cas-3 expression levels decreased *(Fig. 3)* ($P<0.05$).

Discussion

Acute mesenteric ischemia (AMI) are cases that require emergency intervention. AMI injuries are serious and often result in death in late diagnosis. A reperfusion injury that develops following intestinal ischemia causes systemic multi-organ damage, although blood is re-flowed to the intestines with urgent interventions [21]. Therefore, the aim should be the prevention of reperfusion injuries as well as ending ischemia, in the treatment of AMI.

Tissue oxygenation decreases in intestinal ischemia, leading to the initiation of a series of pathological events. During these events, Adenosine Triphosphate (ATP) production stops and cellular respiration begins to be provided by anaerobic respiration. However, by-products of anaerobic respiration accumulate in the cell and cause increased intracellular acidosis. The resulting low pH initiates ischemic damage by causing protein denaturation, loss of enzyme function, glial edema and increased free radicals in the cell [22]. With the re-flow of blood to the tissue, toxic metabolites that occur during ischemia create more aggressive super-

oxide radicals, thus leading to aggravation of tissue damage [23]. On the other hand, intracellular antioxidant systems such as SOD, and GSH are insufficient during ischemia and reperfusion. The cell remains vulnerable to oxidative damage, resulting in increased capillary permeability. Thus, intestinal edema, fluid accumulation in the intestinal lumen, mucosal barrier disruption and bacterial translocation occur [24].

The important indicator of oxidative stress in the tissue is MDA, which is the end product of lipid peroxidation [25]. Oxidative damage has been associated with the measurement of this product in many ischemia and reperfusion studies. In our study, elevated MDA levels in the I and I/R groups support these results. In addition, the low levels of GSH, an intracellular antioxidant, in the I and I/R groups also support our findings. This relationship also appears at the gene level in our molecular analysis findings of GPX and SOD. Another consequence of increased intracellular oxidative damage is the initiation of the intracellular apoptotic caspase cascade. The increased caspase activation can inform us about the severity of oxidative damage. For this purpose, the expression levels of Cas-3 that have an important function in the caspase cascade, prove to us the severity of the oxidative damage in the I and I/R groups at the gene level. We observed the loss of tissue integrity in villus structures in these groups in our histopathological analysis. These results support our findings of high MDA, Cas-3, and low GSH, SOD.

A disrupted mucosal barrier during intestinal ischemia and reperfusion leads to the onset of systemic inflammation and TNF-α is an important signal protein this onset. TNF-α triggers inflammation and increases the release of other inflammation-related cytokines, which lead to excessive migration of neutrophils into ischemic tissue. Neutrophil infiltration leading to increased tissue damage accelerates the destruction of damaged cells in the tissue. Indeed, high levels of TNF-α in the I and I/R groups in molecular analysis findings support this relationship. In addition, increased inflammation and edema findings in our histopathological findings in the I and I/R groups reveal the presence of inflammation. This explains the increase in TNF-α levels.

When looking at the studies on this subject in the literature, high MDA, Cas-3, and TNF-α in the intestinal ischemia/reperfusion study conducted by Kamel et al. were found to be similar to our findings [26]. In another study, low GSH, SOD and GPX findings in ischemia and reperfusion groups also support our study [27].

Red dragon fruit has medicinal therapeutic potential due to its flavonoid, thiamine, niacin, pyridoxine, cobalamin, betalain, phytoalbumin, carotene, polyphenol, and phenolic content [28]. In another study, they investigated the content of Red dragon fruit, showed that it contains a significant amount of antioxidants, and suggested that this fruit may scavenge against free radicals [10]. In our study, we observed significant decreases in MDA levels depending on the dose in the I and I/R treatment groups and significant increases in GSH and GPX levels. In addition to this, we observed significant decrease in Cas-3 levels in the I and I/R treatment groups; these results were also reflected in our histopathological images. Especially, loss of tissue integrity improved significantly in the treatment groups. Another important detail is a decrease of inflammatory cells in the tissue. This result is consistent with the significant decrease in TNF-α gene expression. It has been reported DFE increases MDA levels and GPX levels against oxidative damage [29]. These results support our findings in our study.

Acute mesenteric ischemia followed by reperfusion injury causes severe oxidative stress in the tissue. Reperfusion application alone is insufficient to eliminate oxidative damage but dragon fruit extract can be used as a supportive and protective measure in minimizing this damage to tissue. These results suggest that the content of the dragon fruit needs to be studied in greater detail and that perhaps DFE will be accepted as a preservative supplement food in the near future.

Acknowledgements

Thanks to Oguzhan Kilic for sending fresh fruits we used in the study.

Financial Support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

We declare that there is no conflict of interest between the authors in this article.

Author Contributions

LS contributed to literature searches, study design. ET contributed to critical revision. MY, NAC and SY contributed to methodology. PAK, AH and CO contributed to data acquisition.

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Kafkas Universitesi Veterinar Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 583-588, 2021
DOI: 10.9775/kvfd.2021.25907

RESEARCH ARTICLE

In Vitro and *In Vivo* Anticoccidial Effects of Butyric Acid and Its Impact on Blood and Serum Chemistry of Broiler Chickens

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Article ID: KVFD-2021-25907 Received: 17.04.2021 Accepted: 07.08.2021 Published Online: 08.08.2021

Abstract

Present study was aimed to evaluate the anticoccidial activity of butyric acid by both in vitro and *in vivo* methods. *In vitro* trial was conducted by sporulation inhibition assay to examine the influence of butyric acid on sporulation and damage of coccidian oocysts. Administration of various concentrations of butyric acid induced sporulation inhibition and also damaged coccidian oocysts in dose dependent manner. For *in vivo* trials, 72 (day-old) broiler chicks were randomly divided into 6 groups i.e., A, B, C, D, E and F having equal chicks in each group (n=12). After one week of acclimatization, three doses of butyric acid 1.2%, 1% and 0.8% were given to group A, B and C, respectively while group D was named as positive control (infected medicated), group E was named as negative control (infected and non-medicated) and group F served as normal control (non-infected) group. On the same day, all treated groups were orally infected with 50.000 sporulated oocysts of *Eimeria tenella*. Results revealed that administration of butyric acid induced positive effect on chicken's performance such as weight gain, FCR and anticoccidial parameters like lesion and oocysts score, oocyst per gram. Butyric acid also improved hematological values and serum chemistry of broiler chicken.

Keywords: *Butyric acid, Eimeria, Treatment, Anticoccidial effect, Broiler, Chicken*

Butirik Asitin Etlik Piliçlerde *In Vitro* ve *In Vivo* Antikoksidiyal Etkinlikleri ve Kan ve Serum Kimyası Üzerine Etkisi

Öz

Bu çalışmada, bütirik asitin antikoksidiyal aktivitesinin *in vitro* ve *in vivo* yöntemlerle değerlendirilmesi amaçlandı. Bütirik asitin, koksidian ookistlerin sporülasyonu ve hasarı üzerine etkisini incelemek için *in vitro* sporülasyon inhibisyon testi yapıldı. Çeşitli konsantrasyonlarda bütirik asit uygulanması sporülasyon inhibisyonuna neden olurken, doza bağlı bir şekilde koksidian ookistlere zarar verdi. *In vivo* denemeler için 72 adet bir günlük etlik civciv, her grupta eşit (n=12) civciv olacak şekilde rastgele 6 gruba, yani A, B, C, D, E ve F'ye ayrıldı. Bir haftalık uyum aşamasından sonra grup A, B ve C'ye sırasıyla %1.2, %1 ve %0.8 bütirik asit verilir iken, grup D pozitif kontrol (enfekte ve ilaçlı), grup E negatif kontrol (enfekte ve ilaçsız) ve grup F normal kontrol (enfekte olmayan) grubu olarak değerlendirildi. Aynı gün, tedavi edilen tüm gruplar, *Eimeria tenella*'ya ait 50.000 sporlu ookist ile oral yolla enfekte edildi. Sonuçlar, bütirik asit uygulamasının piliçlerin kilo alımı ve FCR gibi performans ve lezyon ve ookist skoru ve gram başına ookist miktarı gibi antikoksidiyal parametreler üzerine olumlu etkilere yol açtığını ortaya koydu. Bütirik asit ayrıca broyler piliçlerin hematolojik değerlerini ve serum kimyasını da iyileştirdi.

Anahtar sözcükler: *Butirik asit, Eimeria, Sağaltım, Antikoksidiyal etki, Broiler, Tavuk*

How to cite this article?

Rani Z, Abbas RZ, Abbas A, Saeed Z, Rehman T, Hussain, Mehmood K, Rehman A, Hussain K: *In vitro* and *in vivo* anticoccidial effects of butyric acid and its impact on blood and serum chemistry of broiler chickens. *Kafkas Univ Vet Fak Derg,* 27 (5): 583-588, 2021.
DOI: 10.9775/kvfd.2021.25907

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Introduction

In Pakistan, poultry sector contributes to enhance the economy of country by generating employment for about 1.5 million people as well as in meat production sectors contributing 19% of the total meat [1-3]. However, many factors especially diseases play an important role in limiting the poultry production [4,5]. Commercially, poultry farms dealing with broiler chickens are facing many parasitic disorders including coccidiosis, caused by species of *Eimeria* which are obligate protozoans that invade intestinal lining of chicken [6,7], causing severe gastrointestinal damages leading to high mortality rates [8,9]. Moreover, these infected sporozoites induce severe damages like loss of body weight, hemorrhage feces, reduced efficacy and ultimately death of the chicken resulted in serious worldwide economic losses approximately more than 3 billion US dollars [10-12].

Since a long time, many unique anticoccidial drugs are being used in order to treat coccidiosis and their persistent use has caused the drug resistance factor in the birds [13,14]. Due to anticoccidial drug resistance, researchers are trying to find alternative strategies to control coccidiosis in chickens [15-17]. Among alternatives, different acids have shown to improve intestinal health and excellent anti-coccidial effects. Short-chain fatty acids such as butyrate are considered as potential alternatives to antibiotic growth promoters [18,19]. Butyric acid (butyrate) has potential to improve intestinal health [20] and growth stimulant along with bactericidal agent because its activity increases in undissociated form [21,22]. Hence, current study was designed to evaluate the *in vitro* and *in vivo* anticoccidial effects of butyric acid (butyrate) in broiler chickens.

Material and Methods

Acid Collection and Verification

Butyric acid was obtained from an authentic chemical company (Sigma-Aldrich, Pakistan) and was further verified and compared with already stored samples in the Department of Chemistry, University of Agriculture Faisalabad, Pakistan.

Parasite

Eimeria tenella oocysts were collected from the cecal portion of chicken guts and kept in 2.5% sodium hypo-chlorite in the ratio of 4:1 for the time interval of 25 min. Standard method of sedimentation was used for isolation of *E. tenella* oocysts. During sedimentation, desiccators were used and filled with water for four times to get the final results for the evacuation of the coccidial oocysts. Further sporulation of isolated oocysts was done in 2.5% potassiumdichromate solution at 25-29°C and by maintaining 60-80% humidity [23]. The sporulation of the oocyst was confirmed by examining sporocysts under light microscope at 40X.

In Vitro Trial

In vitro experimentation was conducted by sporulation inhibition assay (SIA) to examine the influence of butyric acid against the sporulation of coccidian oocysts. For this purpose, unsporulated oocysts were maintained in petri dishes filled with 2.5% solution of potassium dichromate followed by the thickness of 6 mm and exposed to six different concentrations of butyric acid (10, 5, 2.5, 1.25, 0.625 and 0.31% w/v) in three replicates. Potassium dichromate solution and dimethyl sulfoxide (DMSO) were kept as the control groups. Unsporulated oocysts were incubated at 25-29°C with butyric acid for 48 h. After incubation, tap water was used to wash and then kept at 4°C before counting. The percentage of sporulation was calculated by counting sporulated and non-sporulated oocysts out of 40 oocysts. The effect of butyric acid on morphology of *E. tenella* oocysts was also examined in terms of size and shape. The cover slip was pressed slightly to keep the oocysts pressured for better illustration of their morphology under the microscope.

In Vivo Trial

Seventy-two days old broiler chicks (Hubbard Al-Noor Chicks, Pvt) were purchased from local hatchery and raised under controlled management practices with division into 6 groups having A-C treated with butyric acid 1.2% or 1% and 0.8% through diet (12 chicks in each group) and fed with coccidian free diet, Group D (positive control) was maintained as infective and treated control; Group E (negative control) was maintained as infective and non-treated control while Group F (control) was maintained as non-infective and non-treated control. At the same day, all treated groups were orally infected with 50.000 sporulated oocysts of *E. tenella*. Chicks were subjected to vaccination against New Castle Disease (ND) at 5th day while the vaccination for Infectious Bursal Disease (IBD) was administrated on 8th and 16thday. During one week of acclimatization, chicks were kept at 27-32°C with reduction in each week along with standard light conditions for 24 h.

Anticoccidial Parameters

Anticoccidial activity of butyric acid was assessed by following parameters including:

- Weight Gain and Feed Conversion Ratio

Weekly weight change was assessed from each group until the completion of the experiment. Feed Conversion Ratio (FCR) of birds was calculated by following formula.

FCR = Mean Feed Consumption/Mean Weight (g)

- Lesion and Oocyst Score

On 7th day post inoculation lesion scoring technique was used for that score on a scale of 0 to 4 [23]. At 7th day of

infection, oocysts were scored on a scale of 0 to 5 was done by following the already described method [24].

- Oocyst Per Gram of Feces (OPG)

McMaster technique was used to analyze oocyst per gram of fecesby mixing 3 g of feces with 42 mL of saturated NaCl in beaker (Pyrex) [25]. The oocyst per gram feces (OPG) was calculated by the formula given below.

OPG = oocyst count - dilution factor (fecal sample volume/ counting chamber volume)

Hematological Analysis

Packed cell volume determinations (PCV), red and white blood cell count and haemoglobin (Hb%) level were assessed by using hematology analyzer FMI-6180 (Jiangsu, China) by following the standard method as reported previously [26].

Serum Analysis

Commercially available kits (Fortress Diagnostic Ltd. UK) were used to analyze the serum biochemical parameters such as Alanine transferase (ALT), Aspirate aminotransferase (AST), Urea, Creatinine, Lactate dehydrogenase (LDH) by following standard method provided by the supplier.

Statistical Analysis

ANOVA (analysis of variance) was conducted on different parameters. Furthermore, Duncan's multiple range test was performed to evaluate the significance for the groups. P<0.05 was reported as statistically significant.

Results

The statistical analysis of all treated groups presented that administration of various concentrations of butyric acid significantly affected the sporulation process of *Eimeria* oocysts as compared to control groups (P<0.05) *(Table 1)*.

Butyric acid was also effective in damaging internal and external morphology of *Eimeria* oocysts in concentration-dependent manner as compared to both control groups (P<0.05) as indicated in *Table 2*.

Effect of butyric acid on body weight gain of all treated groups was significantly different to infected non medicated group (P<0.05). Administration of butyric acid also improved FCR of all the treated groups. Statistical analysis of FCR was not conducted due to group feeding of birds, presented in *Table 3*. Administration of various doses of butyric acid significantly reduced oocysts per gram of feces (OPG)and lesion scores which were significantly different to infected non medicated group (P<0.05). The results are shown in *Table 4*.

Table 1. *Efficacy of various concentrations of butyric acid on sporulation of oocysts (%)*

Groups	Treatments (%)	Sporulation (%)
Control-I	0.00	75.00±2.517^{b}
Control-II	0.00	80.00±2.082^{a}
BA (10%)	10.0	20.00±1.155^{f}
BA (5.00%)	5.00	30.00±1.000^{e}
BA (2.50%)	2.50	47.90±1.528^{d}
BA (1.25%)	1.25	48.00±1.000^{d}
BA (0.62%)	0.62	55.00±1.732^{c}
BA (0.31%)	0.31	58.65.00±0.665^{c}

BA: Butyric Acid. Means that bear different letters are statistically significant (P<0.05)

Table 2. *Efficacy of various concentrations of butyric acid on damage of oocysts (%)*

Groups	Treatments (%)	Damage (%)
Control-I	0.00	0.00±0.00^{e}
Control-II	0.00	0.00±0.00^{e}
BA (10%)	10.0	40.30±0.88^{d}
BA (5.00%)	5.00	50.32±1.20^{c}
BA (2.50%)	2.50	58.60±1.45^{b}
BA (1.25%)	1.25	60.00±0.57^{b}
BA (0.62%)	0.62	65.65±0.88^{a}
BA (0.31%)	0.31	68.00±1.15^{a}

BA: Butyric Acid. Means that bear different letters are statistically significant (P<0.05)

Table 3. *Efficacy of various concentrations of butyrate on weight gain and FCR*

Groups	Body Weight (g)	FCR (g/g)
A	355±3.21^{c}	2.6
B	377±4.35^{b}	2.5
C	387±4.35^{ab}	2.1
D	402 ±5.50^{a}	2.4
E	292±4.66^{d}	2.8
F	385±5.19^{ab}	2.2

Means that bear different letters are statistically significant (P<0.05)

Table 4. *Effect of various concentrations of butyric acid on OPG and Lesion score*

Groups	OPG *	OPG **	Lesion Score
A	77.60±1.9^{b}	50.82±3.14^{b}	3.00±0.57^{ab}
B	70.39±1.5^{bc}	45.86±0.87^{bc}	2.33±0.33^{bc}
C	65.71±1.4^{c}	39.40±1.05^{c}	1.33±0.33^{cd}
D	64.21±2.2^{c}	39.48±1.38^{c}	1.00±0.00^{de}
E	95.69±3.4^{a}	75.73±2.51^{a}	4.00±0.57 ^{a}
F	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}

***A:** 1.2%; **B:** 1.00%; **C:** 0.8%; **D:** Infected medicated; **E:** Infected non-medicated; **F:** Normal; **OPG*:** Oocysts per gram of feces 7th day post infection; **OPG**:** Oocysts per gram of feces 14th day post infection*

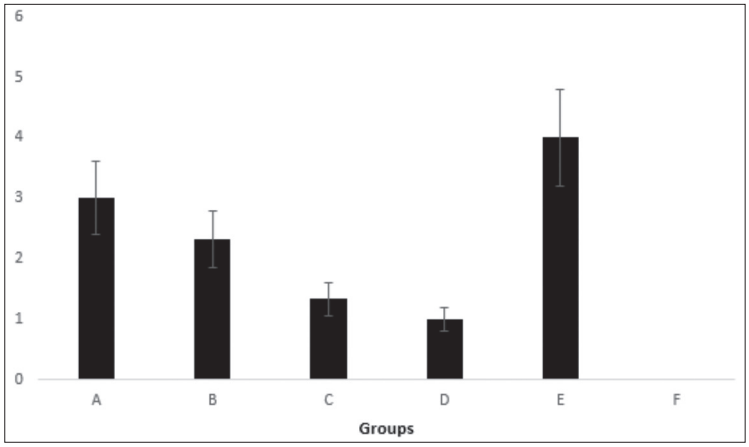
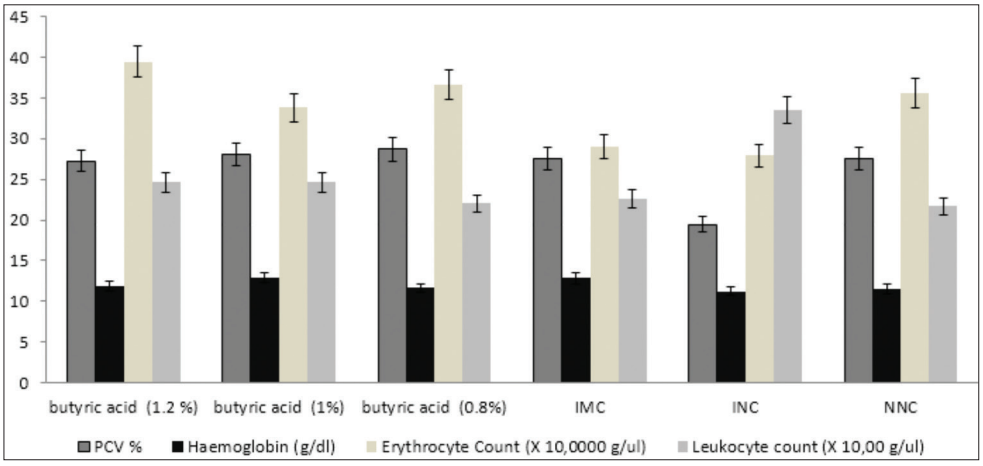


Fig 1. Effect of various concentrations of butyric acid on oocysts score of chickens mixed with *Eimeria tenella*

A: 1.2%; **B:** 1.00%; **C:** 0.8% **D:** Infected Medicated; **E:** Infected non-Medicated; **F:** Normal

Fig 2. Effect of various concentrations of butyric acid (butyrate) on various blood parameters of broiler chicken



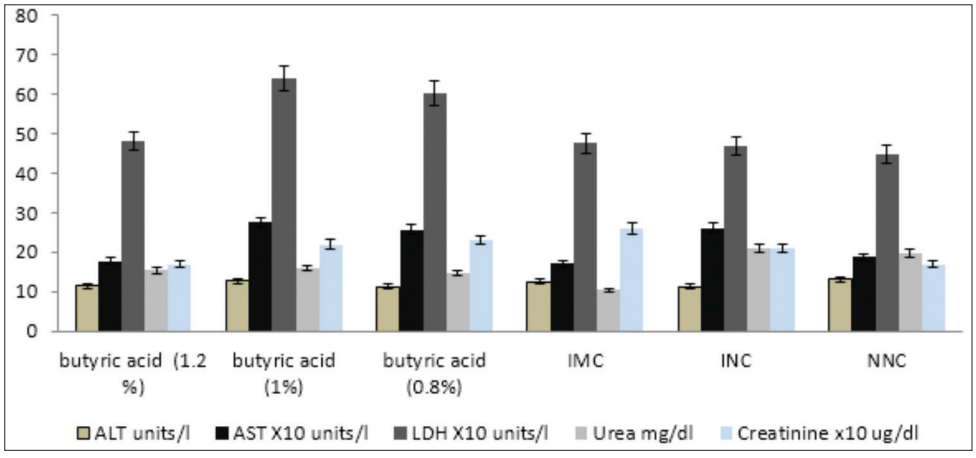


Fig 3. Effect of various concentrations of butyric acid (butyrate) on various serum parameters of broiler chicken

Oocyst score in butyric acid treated groups was also significantly different to infected non medicated group ($P<0.05$) as indicated in *Fig. 1*. Current research studied the effect of various doses of butyric acid on the hematological parameters i.e., Hb, PCV, WBCs and RBCs of broiler chicken mixed with *E. tenella*. Butyric acid treated groups improved hematological parameters which were significantly different to infected non medicated group ($P<0.05$), the results are presented in *Fig. 2*. The effect of various doses of butyric acid on serum enzyme like ALT, AST, urea, creatinine and LDH were significantly different ($P<0.05$) from other groups *(Fig. 3)*.

Discussion

Many recent and previous studies have demonstrated remarkable anticoccidial and growth promoting effects of various organic acids and other novel compounds [27-29] against avian coccidiosis [2,7,8,13,14,30]. Current study has demonstrated *in vitro* and *in vivo* anticoccidial effect of butyric acid. Butyric acid showed inhibitory effect on sporulation process and also damaged *Eimeria* oocyst. Similar types of findings have beenreported in a recent study in which *Vitis vinifera* extract showed *in vitro* anticoccidial

activity in dose dependent manner [6]. The administration of various doses of butyric acid in diet improved weight gain and feed conversion ratio of infected chickens. Positive effect of different organic acids like butyric acid on weight gain and feed conversion of infected birds could be due to their complementary synergistic effect resulting from supplementing diets with a mixture of these additives, especially under the stressful conditions imposed by *Eimeria*. The reported results are in line with previous studies [16-18].

Moreover, in the present research, treatment with various doses of butyric acid induced protective effect by lowering oocysts per gram of feces (OPG), lesion and oocyst score in broiler chicken. Likewise, the different concentrations of acetic acid reduced negative performance and pathogenic effects (lesion and oocyst score) associated with *E. tenella* challenge in broiler chickens [28]. In another study, similar type of anticoccidial effect of hydrochloric acid (HCl) has been reported. Administration of hydrochloric acid in water reduced lesion and oocyst score against *E. tenella* infection in broiler chickens and also improved weight gain and feed conversion ratio of birds [29].

Different organic acids have been reported for their positive effect on performance parameters and have also shown therapeutic effects against coccidiosis. Previously, dietary supplementation of 0.2% butyric acid at the concentration of 0.2% improved the growth performance and carcass quality of broiler chicks experimentally challenged with coccidiosis [30]. Different studies are warranted on the effects of butyrate on epithelial cell development in the small intestine of young broilers and suggested butyrate the best candidate for improving performance of broiler chickens [31]. Likewise, antimicrobial and antibacterial activities of various acids as additives to enhance the protective effect of anticoccidial drugs have also been reported in broiler chickens [32,33].

Findings of current studies are similar with the research outcomes of previous studies [34,35] who found similar effect of butyric acid on serum chemistry of broiler chickens. Moreover, present findings of butyric acid on serum enzymes levels (ALT and AST) were also observed in previous studies [34,35]. Hematological parameters were also improved in infected birds as reported previously [36] where similar trend of butyrate effect on red and white blood cells count was observed in broiler chicken.

In a recent study, maslinic acid found in plant *Olea europaea* showed the remarkable increase in weight gain and reduced the lesion score in chickens infected with *E. tenella*. Histopathological studies showed a significant decrease in the infection rate in the chicks treated with maslinic acid [37].

In one study, the three products of organic acids including Acidomix® (ammonium formate, formic acid, ammonium propionate), Activate® (calcium butyrate, fumaric acid, benzoic acid) and Lacplus® (lactic acid, citric acid, fumaric acid) induced protective immunity, improved intestinal health and reduced lesion and oocyst score against *E. tenella* infected broiler chickens [38].

Most recently, butyric acid (tributyrin) has been reported to improve weight gain and immunity against *Eimeria maxima* infected broiler chicken [39]. Additionally, dietary supplementation of formic acid (0.5%) and propionic acid (0.5%) produced positive effects in terms of growth performance, gut microbiota and pH, carcass traits, and immune response in broiler chickens. Dietary supplementation with formic or propionic acids positively influenced the production parameters, but a mixture of both acids produced more effects in broiler chickens [40].

Present research concluded that treatment with different doses of butyric acid induced protective effect on body weight, lesion score, FCR value and oocysts per gram of feces. Moreover, induction of butyric acid also induced dose dependent protective effect on hematological parameters (RBCs, WBCs, Hb, PCV) and serum chemistry (ALT, AST, Urea, Creatinine, LDH) in the *E. tenella* infected chickens. However, further research is needed for the development of acid based anticoccidial product especially from butyric acid.

Author Contributions

ZR, RZA, AA, ZS planned and designed the research. TR, RH, KM helped in execution of the research trials, data collection and analysis. AR and KH assisted in hematology and serum chemistry.

Conflict of Interest

The authors declared that there is no conflict of interest between the authors.

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Kafkas Universitesi Veterinar Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 589-594, 2021
DOI: 10.9775/kvfd.2021.25921

RESEARCH ARTICLE

Atractyloside Levels in *Xanthium strumarium* and Atractyloside Concentrations in the Serum of Rats Given *Xanthium strumarium*

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Article ID: KVFD-2021-25921 Received: 20.04.2021 Accepted: 12.09.2021 Published Online: 16.09.2021

Abstract

Atractyloside (ATR) may cause severe liver and kidney damage. However, there is a lack of information about levels of ATR in parts of Xanthium strumarium and the concentrations and kinetics of ATR in the serum. In this study, we determined atractyloside levels in *Xanthium strumarium* parts, its serum concentrations and some kinetic parameters concerning time with the gas chromatography-mass selective (GC-MS) method. After administering (80 mg/kg ATR) *X. strumarium* seed extract to rats through gastric gavage, blood samples were collected at the 0th, 4th, 6th, 8th, 12th, 24th, 36th, 48th, 60th, 72nd, 96th, and 120th h. After extraction, hydrolysis and derivatization of the plant and serum samples were analyzed by the GC-MS instrument. The ATR level in the *X. strumarium* seeds was 3.043 mg/g in August, 3.502 mg/g in September and 3.800 mg/g in October. ATR was not detected in other parts of the plant. After ATR administration to rats, the C_{max} value of ATR was calculated as 10.77 µg/mL at T_{max} of 48 h and $t_{1/2}$ of 6.07 h. A thorough understanding of ATR circulation in the blood will aid in determining the course of its toxic effects in the bloodstream, the onset of symptoms and the general management plan for ATR poisoning. Moreover, the results obtained from this study will contribute to the antidote studies for ATR poisoning.

Keywords: *Atractyloside, GC-MS, Rat, Serum, Xanthium strumarium L.*

Xanthium strumarium'da Atraktilozid Seviyeleri ve *Xanthium strumarium* Verilen Sıçanların Serumunda Atraktilozid Konsantrasyonları

Öz

Atraktilozid (ATR) karaciğer ve böbrekte ciddi hasarlar yapabilen bir toksindir. Buna karşın bileşiğin kandaki dağılımı ve kinetiği hakkındaki bilgiler yetersizdir. Bu çalışmada gas kromatografi-kütle spektrofotometre (GC-MS) ile *Xanthium strumarium*'daki atraktilozid seviyelerini, serum konsantrasyonunu ve zamana bağlı bazı farmakokinetik parametrelerini belirledik. Sıçanlara (80 mg/kg ATR) *X. strumarium* tohum ekstresinin mide sondası ile uygulanmasından sonra 0, 4, 6, 8, 12, 24, 36, 48, 60, 72, 96, ve 120. saatlerde kan alındı. Serum ve bitki örneklerinin ekstraksiyonu, hidrolizi ve türevlendirilmesi yapıldıktan sonra GC-MS cihazı ile analizi yapıldı. *X. strumarium* tohumlarında ATR seviyesi Ağustos'ta 3.043 mg/g, Eylül'de 3.502 mg/g ve Ekim'de 3.800 mg/g saptanmıştır. Bitkinin diğer kısımlarında ATR saptanmamıştır. ATR'nin C_{max} değeri 10.77 µg/mL, T_{max} 48 saat ve $t_{1/2}$ 6.07 saat olarak hesaplandı. Kandaki ATR seviyesinin dağılımının bilinmesi zehirlenmelerde semptomların başlangıcını, toksik etkilerin seyrini ve sağaltım planını belirlemeye yardımcı olacaktır. Ayrıca, bu çalışmadan elde edilen bulgular, ATR zehirlenmesi ile ilgili antidot çalışmalarına katkı sağlayacaktır.

Anahtar sözcükler: *Atraktilozid, GC-MS, Serum, Sıçan, Xanthium strumarium L.*

INTRODUCTION

Xanthium strumarium L. (Cocklebur) belonging to the Asteraceae family, which is poisonous and common in many parts of the world, is an annual plant species. It is 20-90 cm in height, its leaves are green, and each fruit of

How to cite this article?

Ozturk Y, Keskin Z, Tanyildizi S, Baykalir BG, Korkak FA, Dagoglu Hark B, Dagoglu G: Atractyloside levels in *Xanthium strumarium* and atractyloside concentrations in the serum of rats given *Xanthium strumarium*. *Kafkas Univ Vet Fak Derg,* 27 (5): 589-594, 2021.
DOI: 10.9775/kvfd.2021.25921

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this plant is 1-3.5 cm long brown, hard, woody and hook-shaped thorny fruits contains two seeds [1,2].

Xanthium species have been used as traditional herbal medicines for centuries in Eastern countries [3,4]. The whole plant has been used to treat bacterial infections, skin pruritus, diabetes, rhinitis, rheumatoid arthritis, as well as for its analgesic, anti-inflammatory, antitumor and cytotoxicity activity [5-8].

Atractyloside (ATR) is a compound found in *X. strumarium* that leads to poisoning in humans and animals. ATR-induced intoxication could be lethal to pigs, sheep, cattle and humans. In animals, *X. strumarium* toxicity causes fatigue, weak pulse, nausea, ataxia, convulsion, depression, anorexia, vomiting, dyspnea, abdominal pain, weakness and recumbency resulting in death between 6 and 96 h after ingestion [5,9]. The liver toxicated by ATR shows the most distinctive microscopic lesions, which are characterized by the acute diffuse necrosis of the centrilobular hepatocytes accompanied by congestion and hemorrhage [10-13].

By preventing oxidative phosphorylation in cells, ATR leads to the disruption of the energy metabolism, opening of mitochondrial permeability transfer pore (mPTP) channels and necrotic/apoptotic cell death [12,14-16].

In this study, ATR levels found in the *X. strumarium* and some kinetic parameters of ATR given rats through gastric gavage were investigated. Determining ATR levels in *X. strumarium* and its kinetics in rat poisoned by will contribute to determining the course of intoxication and treatment methods.

Material and Methods

Ethical Statement

Ethics committee approval for this experimental study was obtained from Bingol University Animal Experiments Local Ethics Committee, with the decision dated 21.02.2018 and numbered 02/01.

Animals

This study included a total of 72 male Wistar Albino rats (6-7 weeks old, 200-250 g) with six animals in each group. The experimental animals used in the study were obtained from Bingol University, Experimental Research Center. All rats were housed in special cages under standard conditions (at a fixed temperature and in ventilated rooms; 12 h of daylight and 12 h of dark). Fresh water and feed were given to the rats *ad libitum*.

Reagents and Materials

Atractyloside (potassium salt) was obtained from Cayman (Cas no: 102130-43-8, purity ≥95), whereas trimethylsilyl imidazole (TMSI), pyridine, ethyl acetate, acetone and other chemicals to be used in the GC-MS analysis were purchased from Merck (Darmstadt, Germany).

Preparation of Stock Standard Solution

Atractyloside (potassium salt) was prepared in a methanol solution, and the standard solution was stored at +4°C. The concentration of ATR was 12.5 mmol/L.

Preparation of X. strumarium Extract Applied to Rats

The plant material was collected from the Elazig region in October, and the seeds were taken out of the fruits. A 100 g amount of *X. strumarium (*voucher number*:* Soberats, TR9008, CIFMT, Fuentes, 4785, ROIG) of seed was weighed with a precision scale and heated-refluxed twice with purified water (1:5, w/v) for 2 h each time. The extract was then filtered, combined and concentrated under reduced pressure in a vacuum rotary evaporator. The contents of the extract were measured using the GC-MS method, and it was found to contain 10 mg/mL of ATR. Due to the lack of dose studies of ATR in the literature, nontoxic 80mg/kg dose of ATR used in this study. That amount of dose is inferentially determined on the base of Bouabid et al.[17] study's results about oral LD_{50} dose of 1000 mg/kg. 80 mg/kg dose of ATR was given to the rats as *X. strumarium* seed extract via oral.

Preparation of Serum Samples

An aliquot of 0.5 mL of rat serum sample was spiked with 1 mL of acetone placed and vortexed. The mixture was then centrifuged (3500xg. 5 min, 4°C), and the supernatant was removed [18]. The supernatant was dried under a stream of N_2 at 40°C. The dried extract was reconstituted with 1 mL distilled water and acidified with 2 mL of hydrochloric acid (2 mol/L). The tubes were vortexed and stored overnight. The hydrolysates were extracted five times with 2 mL ethyl acetate. The combined organic extracts were dried under a stream of N_2 at 40°C. The dried extract was reconstituted with 200 µL of TMSI, and 200 µL of pyridine was added. Derivatization was performed at 100°C for 2 h before the GC-MS analysis [19].

GC-MS Conditions

A Shimadzu GC-MS device equipped with a Shimadzu auto-injector and a Shimadzu mass-selective detector was used for the GC-MS analysis (Shimadzu, GCMS-QP2010) with a DB-1 capillary column (30 mx250 µm I.D., film thickness: 0.1 µm).

For injection in the pulsed splitless mode, the injector temperature was 250°C, the ion source temperature was 200°C, the helium carrier gas flow rate was 1.9 mL/min, and the oven temperature was programmed to rise from 215 to 310°C at 2.30/min. The run time was 45 min under these conditions.

Preparation of X. strumarium Extract

Xanthium strumarium samples were collected from the Elazig

region from May to October. ATR levels were measured in the roots, stems, leaves and seeds of *X. strumarium* from May to October while the plant was growing. One g amount of *X. strumarium* seeds (Voucher number: Soberats, TR9008, CIFMT, Fuentes, 4785, ROIG; authenticated by the Department of Biology, Firat University) was powdered and extracted in 10 mL water at 100°C for 15 min. 1 mL of the plant extract was acidified with 2 mL of hydrochloric acid (2 mol/L). The extract samples were vortexed and stored overnight. The hydrolysates were extracted five times using 2 mL ethyl acetate. The combined organic extracts were dried under a stream of N_2 at 40°C. 200 µL of TMSI and 200 µL of pyridine were added to the dried extracts, and derivatization was performed at 100°C for 2 h. Then, 2 µL of derivatives were injected into the GC-MS device [19].

Bioanalytical Method Validation

The method was validated in the whole serum and plants according to the FDA Bioanalytical Method Validation Guidance for Industry (2018 edition).

Selectivity, Specificity, and Carryover

Selectivity was assessed by comparing blank rat serum from six individual sources, spiked samples with ATR at low limit of quantification (LLOQ) and a real serum sample obtained from *X. strumarium* extract-treated rats. The analyte responses in the blank should be less than 20% LLOQ of spiked samples. Carryover between samples was evaluated in five circles by detecting the blank samples immediately after upper LOQ samples.

Calibration Curve

The calibration curves for ATR were comprised of a blank (no analyte), a zero calibrator, and seven non-zero calibrator levels covering the quantitation range on three consecutive days. The linearity of every calibration curve was obtained by evaluating the concentration-response relationship using a weighted ($1/x^2$) least squares linear regression. Non-zero calibrators should be ±15% of nominal concentrations, except at LLOQs (±20%).

Accuracy and Precision

Evaluating the accuracy and precision across the quantitation range involves analyzing the performance of ATR in a calibration curve and at the LLOQ, low, medium, and high QCs in five replicates per QC level in three independent runs. The precision within-run and between runs (described as RSD%) must be within 15%, except 20% at LLOQ. The accuracy (described as relative error, RE) should not deviate ±15% of nominal concentrations, except ±20% at LLOQ.

Extraction Recovery and Matrix Effect

The recoveries of ATR were calculated by comparing the peak areas between extracted samples with post-extracted spiked samples at low, medium, and high QC concentrations. The matrix effects were determined by the peak-area ratio between post-extraction samples with water-substituted samples at three QC levels

Stability

The stability study was performed at three replicates at low and high concentrations of QC samples including stock solution stability, bench-top stability, autosampler stability, post-preparation stability, freeze-thaw stability, and long-term stability. The method was considered to be stable when the accuracy (% nominal) at each level was ±15%.

Pharmacokinetic and Statistical Study

Blood samples (1 mL) were collected from the orbital venous plexus at 0, 4, 6, 8, 12, 24, 36, 48, 60, 72, 96, and 120 h after the rats received a single intragastric administration of the *X. strumarium* extract. The pharmacokinetic parameters of the samples were calculated using non-compartmental analysis with the PKSolver program. All analyses were performed using IBM SPSS Statistics Version 22.0 statistical software package.

Results

Standard Curve

The standards were prepared at different concentrations from the stock solution, and chromatograms were determined. The linear equation was f(x)=5466X+1771, r^2=0.946 [f(x)= peak area and X=concentration (µg/µL)]. The chromatograms of the ATR standard solution (8 µg) and the rat blood samples are demonstrated in *Fig. 1-A,B*. The retention time was 36.2 min for ATR.

Sensitivity, LOD, LOQ and Recovery

For the method used in the analysis, the limit of detection (LOD) was 17 ng/mL, and the limit of quantification (LOQ) was 50 ng/mL.

Recovery was examined from the QCs for 20 ng/mL, 35 ng/mL and 60 ng/mL concentration ranges in the serum and plant samples. The mean recovery was 89.50±5.1% in the serum samples and 92±6% in the plant samples.

Distribution of ATR in the Serum of Rats

As seen in *Table 1*, after the administration of *X. strumarium* through gastric gavage, the absorption of ATR began at the 6th h, and its presence in the serum remained even at the 96th h. The mean plasma ATR concentrations in all groups are shown in *Table 1*.

Some kinetics parameters obtained after the intragastric administration of the *X. strumarium* extracts to the rats included a peak plasma concentration (C_{max}) of 10.77 µg/mL, and the time for the drug concentration to reach the peak value (T_{max}) was 48 h. Some pharmacokinetic parameters

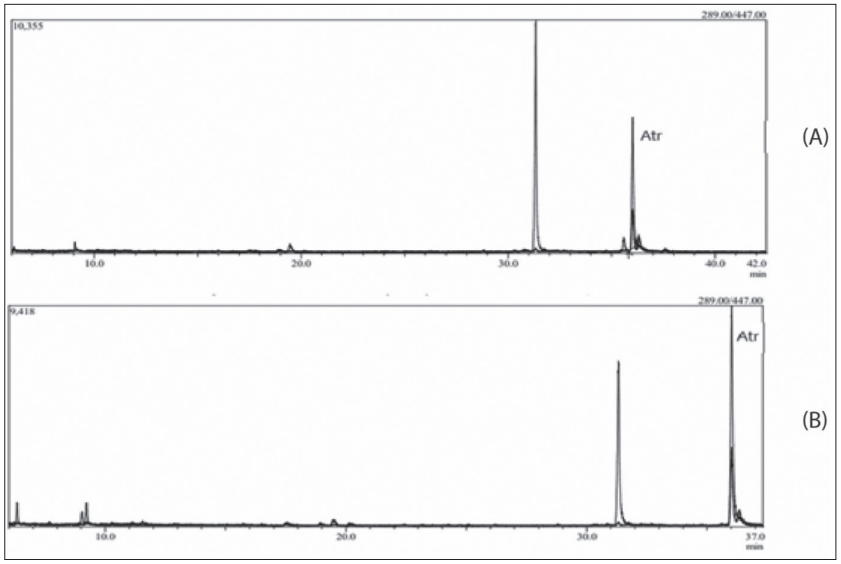

Fig 1. Standard and serum chromatograms. **A-** Chromatogram (standard solution, 8 µg), **B-** Chromatogram (serum sample, 24 h)

Table 1. *Periods of blood drawing and average serum ATR levels (µg/mL, n=6)*

Groups	Time (hour)	Mean±SEM
Control	0	0±0
Group 1	4	0±0
Group 2	6	0.031±0.004
Group 3	8	0.053±0.011
Group 4	12	0.072±0.192
Group 5	24	4.983±1.258
Group 6	36	7.126±1.599
Group 7	48	10.776±2.601
Group 8	60	7.200±1.333
Group 9	72	3.433±0.944
Group 10	96	0.134±0.034
Group 11	120	0±0

Table 2. *Main pharmacokinetic parameters after a single gastric administration of 80 mg/kg ATR (n=6)*

Kinetic Parameters	Unit	Oral ATR
$T_{1/2}$	h	6.071
T_{max}	h	48
C_{max}	µg/mL	10.77
MRT	h	47.807
V_{dss}	L/kg	1.623
Cl	L/kg/h	0.185
AUC	µg/h/mL	430.231

*$t_{1/2}$: elimination half-life, C_{max}: maximum concentration attained, T_{max}: time at which C_{max} is attained, **MRT:** mean residince time, V_{dss}: volume of distrubition at steady state, **Cl:** clearance of drug, **AUC:** total are under the concentration time-curve, **AUMC:** total area under the first moment concentration-time-curve*

of ATR are shown in *Table 2*, and the concentration-time curve of ATR is presented in *Fig. 2*.

Distribution of ATR levels in X. strumarium Parts by Months

ATR was not present in the root, stem or leaves of the plant in May-October as shown in *Fig. 3*. As of August, when the *X. strumarium* plants began to seed, the ATR levels in the seed were tested. In August, the mean ATR concentration in the seeds was 3.043 mg/g, whereas it was 3.502 mg/g in September and 3.800 mg/g in October. In September and October, the ATR concentrations in the seeds were higher than those in August ($P<0.05$, $P<0.001$ respectively). However, there was no significant difference between the values in September and October ($P>0.05$).

Discussion

Ingestion of *X. strumarium* plant contains ATR causes poisoning in humans and other animals. By preventing oxidative phosphorylation in cells, ATR leads to the disruption of the energy metabolism, stimulates the opening of mPTP channels and brings about necrotic/apoptotic cell death. Studies that had concentrated on the ATR levels in blood and various parts of the plant in cases of poisoning in animals are not sufficient. It is believed that the reason for this is that analyzing ATR is difficult, as stated by some authors [20-22].

In the literature, there is no study on ATR concentrations in various parts of the *X. strumarium* plant on monthly basis. In this study, ATR was detected in the seeds of the plant as at 3.01 mg/g in August, 3.4 mg/g in September and 3.9 mg/g in October, while it was not detected in the other parts of the plant. The absence of ATR in leaf, stem and root of the plant, also the existence of ATR in seed correlates with higher levels of ATR in seed in August-September-October months which is the seeding period. In addition to that, the analysis held in November due to the rotting of the plant, ATR levels could not be measured.

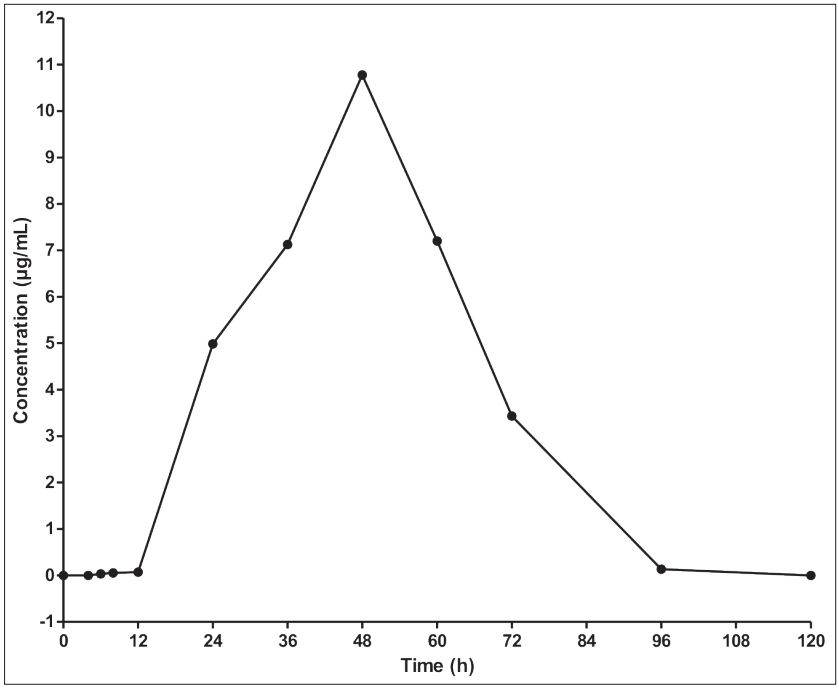

Fig 2. Serum concentration-time curve of ATR

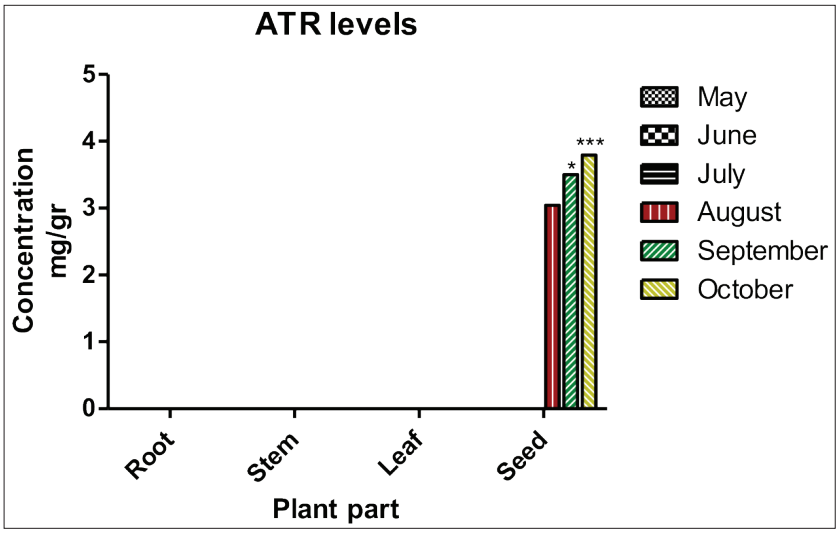

Fig 3. ATR levels of *X. strumarium* by month and plant part. Difference in seed according to August *** P<0.001, * P<0.05

In our study, for ATR, the C_{max} value was found as 10.77 µg/mL, T_{max} was determined as 48 h, and $t_{1/2}$ was calculated as 6.07 h, where the concentration fell below the detection limit after the 96th h. Fan et al.[2] reported the values of $t_{1/2}$ as 13.64, 9.62 and 8.61 h, T_{max} as 0.38, 1.85 and 0.27 h and C_{max} as 41.98, 24.61 and 263.40 µg/mL after administering ATR at the respective concentrations of 11.4, 22.8 and 45.6 mg/kg. Carlier et al.[23] detected 0.883 µg/mL ATR in the blood of a woman poisoned with the *Atractylode gummifera* plant at the end of the 3rd day. The formation of the symptoms of ATR poisoning and the findings of Carlier et al.[23] showed that ATR stays in the bloodstream for a long time. This supported our findings on the T_{max}, C_{max} and $t_{1/2}$ values.

Determining the ATR levels *X. strumarium* and MRT, T_{max}, C_{max}, $t_{1/2}$ and CL etc. values in rat serum will provide valuable information about preventing, diagnosis and treatment of ATR poisoning. The results of this study could enlight the way to discovery of the antidote of *X. strumarium* poisoning and will be a leading source for further investigations.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Financial Support

This manuscript received no grant from any funding.

Conflict of Interest

The authors have declared that no conflicts of interest.

Author Contributions

Tanyildizi S, Dagoglu G, Baykalir BG planned, designed and supervised the research procedure, the samples were collected by Ozturk Y, Keskin Z, Korkak FA performed the analysis, and Hark BD conducted the statistical analysis.

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Kafkas Universitesi Veterinar Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 595-600, 2021
DOI: 10.9775/kvfd.2021.25935

RESEARCH ARTICLE

The Healing Effects of The Topical Mesenchymal Stem Cells Application on Colonic Anastomosis Subjected to Ischemia Reperfusion Injury

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Article ID: KVFD-2021-25935 Received: 23.04.2021 Accepted: 07.08.2021 Published Online: 07.08.2021

Abstract

Intestinal ischemia reperfusion injury (IRI) is a challenging problem and it adversely affects the healing of colonic anastomosis. Our experimental study aimed to investigate the role of mesenchymal stem cells (MSC) administration in the healing of colonic anastomosis. A total of 33 rats were grouped as Control, IRI and MSC treatment groups. Three rats were reserved for obtaining MSCs. Colonic resection and anastomosis procedure was performed in all groups. Anastomotic line was wrapped with MSCs impregnated spongostan after colonic anastomosis in the rats of the MSC treatment group. All rats were sacrificed and anastomotic line were sampled for examination on the post operative seventh day. Tissue hydroxyproline (HP) levels and anastomotic bursting pressures were statistically compared. Anastomotic bursting pressures were found to be significantly high in MSC treatment group rats. The lowest anastomotic bursting pressure was detected in IRI group rats. Hydroxyproline content of the anastomotic sites were also found to be significantly higher in the rats of the MSC treatment group when compared with the IRI group rats. Our study showed that the detrimental effects of IRI on the healing process of colonic anastomosis in an experimental model may be alleviated with the treatment of MSCs.

Keywords: *Anastomotic leakage, Colonic anastomosis, Hydroxyproline, İschemia reperfusion injury, Mesenchymal stem cell*

Topikal Mezenkimal Kök Hücre Uygulamasının İskemi Reperfüzyon Yaralanmasına Bağlı Kolonik Anastomoz Üzerine İyileştirici Etkileri

Öz

Bağırsak iskemi reperfüzyon hasarı (İRH) zorlu bir sorundur ve kolon anastomozunun iyileşmesini olumsuz etkiler. Deneysel çalışmamız kolon anastomozunun iyileşmesinde mezenkimal kök hücre (MKH) uygulamasının rolünü araştırmayı amaçlamıştır. Toplam 33 sıçan, Kontrol, İRH ve MKH tedavi grupları olarak gruplandı. MKH'leri elde etmek için üç sıçan ayrıldı. Tüm gruplara kolonik rezeksiyon ve anastomoz işlemi uygulandı. Anastomotik hat, MKH tedavi grubundaki sıçanlarda kolonik anastomozdan sonra MKH emdirilmiş spongostan ile sarıldı. Tüm sıçanlar ameliyat sonrası yedinci günde sakrifiye edildi ve inceleme için anastomoz hat örneklendi. Doku hidroksiprolin (HP) seviyeleri ve anastomoz patlama basınçları istatistiksel olarak karşılaştırıldı. MKH tedavi grubu sıçanlarda anastomoz patlama basınçları önemli ölçüde yüksek bulundu. En düşük anastomoz patlama basıncı İRH grubu sıçanlarda tespit edildi. Anastomotik bölgelerin hidroksiprolin içeriği, İRH grubu sıçanlara kıyasla MKH tedavi grubundaki sıçanlarda önemli ölçüde daha yüksek bulundu. Çalışmamız, deneysel bir modelde İRH'nin kolon anastomozunun iyileşme süreci üzerindeki zararlı etkilerinin MKH'lerin tedavisi ile hafifletilebileceğini göstermiştir.

Anahtar sözcükler: *Anastomoz kaçağı, Hidroksiprolin, İskemi reperfüzyon hasarı, Kolon anastomozu, Mezenkimal kök hücre*

How to cite this article?

Özden H, Karaca G, Bulut H, Niyaz M, Gömeç M, Daphan ÇE: The healing effects of the topical mesenchymal stem cells application on colonic anastomosis subjected to ischemia reperfusion injury. *Kafkas Univ Vet Fak Derg,* 27 (5): 595-600, 2021.
DOI: 10.9775/kvfd.2021.25935

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Introduction

There may be a need for anastomosis in bowel surgeries that are reperformed quite frequently. It is a type of surgery with high mortality and morbidity that is feared in surgery clinics. A leak that may develop from the anatomosis can be very risky for the life of the patient. Therefore, studies in this field are frequently encountered in the literature. To increase the safety of the anastomosis, many agents are tried systemic and topical. The main problem that we frequently encounter in the deterioration of the well-being of the anastomosis in clinical practice is the oxygenation and nutritional status of the anastomotic line. An ischemic bowel carries a high risk for anastomosis. Reperfusion injury after oxygenation of the ischemic tissue causes tissue damage and prevents healing in the anastomosis line [1].

The effect of ischemia/reperfusion (I/R) injury on the healing of colonic anastomosis is one of the most investigated topics of experimental surgery. Intestinal ischemia may result from many clinics senarios such as mesenteric vascular occlusion, mechanical obstruction, strangulated hernia or volvulus. Shock and severe cardiopulmonary diseases are also common clinic problems and they constitute the cause of a more prevalent but underdiagnosed type of intestinal ischemia [1]. Removing the necrotic colonic segment and performing colonic anastomosis may be required in these conditions. A major indicator of the outcome of this procedure is the safety of colonic anastomosis. Factors including the degree of ischemia, the length of ischemic bowel segments and the performance status in which the patient plays an important role in the anastomotic healing process. It has been shown that the presence of I/R injury on intestinal anastomosis delays the anastomotic healing process and this may lead to anastomotic leakage and dehiscence. Although anastomotic leakage and dehiscence seem to be local events of I/R injury, the mediators from the ischemic tissue enter the systemic circulation and affect to their organ systems [2]. Endothelial dysfunction, increased free radical production, nitric oxide depletion and released cytokines are the main characteristics of the mechanism of I/R ınjury. These events trigger a local and systemic inflammatory response according to the severity of ischemic insult. Endothelial dysfunction and cytokine release are the main unfavorable factors responsible for tissue damage [3].

Ischemia reperfusion injury is generally an unavoidable challenging problem. Investigations in this field have focused on early detection and have examined the effects of therapeutic agents on tissue damage [4].

Mesenchymal stem cells have beneficial effects on anastomotic safety in the digestive tract in the presence of ischemia. MSCs from adipose tissue have immunomodulatory, anti-inflammatory and anti-apoptotic properties [5,6]. We aimed to investigate the healing effects of MSCs on colonic anastomosis subjected to I/R injury in our study.

Material and Methods

Ethical Approval

This experimental study was approved by Kırıkkale University Animal Experiments Local Ethics Committee on 09.01.2014 with the number 14/14.

Animals

A total of 33 rats were grouped as Control, IRI and MSC treatment groups. Three rats were reserved for obtaining MCSs and the others were grouped as control (n=10), I/R injury (n=10) and MCS treatment group (n=10).

Preparation of MSCs Impregnated Spongostan Layers

Mesenchymal stem cells were obtained from subcutaneous adipose tissue in the abdomen of rats. Stem cells were isolated by using the primary culture method. Fat tissue was collected from three appropriately anesthetized rats. An average of 0.59 g fat tissue was collected per rat ($n = 3$; $n^1 = 0.64$ g, $n^2 = 0.54$ g, $n^3 = 0.59$ g). The fat tissue was transported in an appropriate transport medium (transport medium containing 10% FBS and 0.4% penicillin-streptomycin) and incubated in standard culture medium by splitting into small pieces. The culture medium was changed daily to prevent the possible different effects of various cytokines induced by MSCs. Cells were passaged 4 times using standard trypsinization method and the number of cells was counted using trypan blue staining method when they were passaged. They were then frozen for use. Characterization of the cells was performed using flow cytometry. It was analyzed for CD29, CD90, CD54, MHC class 1, CD45, CD109 and MHC class 2 receptor. $9x10^6/mm^3$ MSCs prepared separately for each transport container were impregnated with layers of spongostan [7].

Anesthesia and Surgery

Rats were anesthetized using intraperitoneal ketamine HCl 90 mg/kg (Ketalar, 500 mg/10 mL Pfizer; USA) and 10 mg/kg xylazine HCl (Rompun, 23.32 mg/mL Bayer, Leverkusen, Germany). Operation sites of the rats were cleaned with povidone-iodine before incision. About 3 cm midline incision was performed in all rats. In control group rats, 0.5 cm colonic segment was resected in distance 5 cm from the ileocecal valve and later anastomosis added. As described by Fink et al.[8] superior mesenteric artery was clamped for about 15 min for ischemia and intestinal tissue was evaluated for pallor and edema, and released for 5 min to ensure reperfusion before colonic resection and anastomosis procedure in I/R injury group rats. The presence of ischemia was confirmed by the color changes. MSCs impregnated spongostan layers ($9x10^6/mm^3$) were prepared as mentioned below for the rats of the MSC treatment group. After subjection to I/R injury, resection and anastomosis procedure was performed and colonic anastomotic line was wrapped with MSCs

impregnated spongostan layers in MSC treatment group rats. All rats were allowed standard rat chow and water as before surgery. On the seventh postoperative day, all rats underwent relaparotomy and 5 cm of anastomotic colon segments were removed for the examination of tissue hydroxyproline levels and for measuring anastomotic bursting pressure.

Measurement of Anastomotic Bursting Pressure

The anastomotic bursting pressure was measured in all rats as described in the literatüre [9]. A 5 cm colonic segment (including the anastomosis in the middle) carefully resected and fecal content was cleaned with saline solution. The proximal end of this segment was ligated by using 2/0 polyglactin suture and the other end was fixed to the infusion pump using a 16G catheter and then infused with isotonic saline solution at 2 mL/min. The intraluminal pressure of the colonic segment was monitored and measured from the anastomotic site until a leak occurred and the pressure was recorded as anastomosis burst pressure (BP) *(Fig. 1* and *Fig. 2)*.

Evaluation of Hydroxyproline Level in Perianastomotic Tissue

After the measurement of anastomotic bursting pressure, wet perianastomotic tissue samples were weighed, then dried for 3 days at 60°C. Dry tissue samples were also weighed. The tissues were hydrolyzed in 7 N hydrochloric acid (HCl) at 110°C for 8 h and centrifuged at 5000 rpm for 20 min to obtain the study material. The absorbance of the formed material was evaluated colorimetric (photometric) at 121°C at 562 nm and the tissue hydroxyproline (HP) level was calculated.

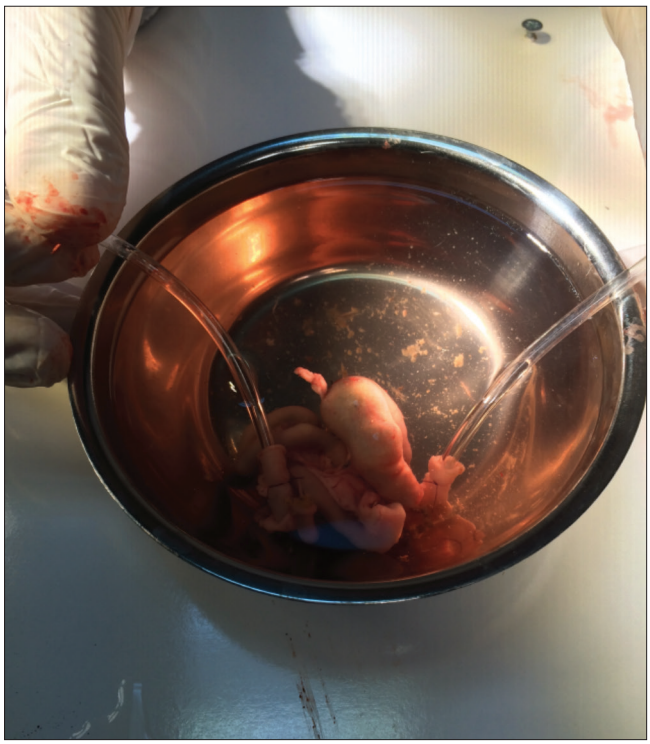

Fig 1. Anastomotic bursting pressure, anastomosis line

Fig 2. Anastomotic bursting pressure, infusion pump

Statistical Analyses

All results are reported as mean ± standard error of the mean. The statistical analyses were performed by using the SPSS® statistical package, version 16.0 for Windows. Due to limited number of rats in each group, non-parametric methods were used for statistical analysis. Kruskal-Wallis variance analysis, which is used to compare the means of three or more groups, was used to determine whether there was a statistical difference between the groups. The Mann-Whitney U test, which is used to compare the means of two groups, was used to determine the origin of the significant difference in terms of groups. P value of less than 0.05 was considered significant.

Results

The experimental protocol was composed of three groups as control, I/R and MSC groups. Ten rats were randomly selected for each group and a total of 30 rats underwent surgery. One rat from the control group, one from the MSC group, and two rats from the I/R group died within the first day after the first surgical procedure of the experiment. Relaparotomy was performed for deceased rats. There was no intra-abdominal adhesion, anastomotic leakage or any

Table 1. *Anastomotic bursting pressure levels of the groups*

Groups	Bursting Pressure		
	Min	Max	Median
Control group	170	230	217.78[a]
I/R group	160	210	199.09[a,c]
MSC group	180	260	236[c]

*Values are presented as median (intrequartile range) *Kruskal-Wallis test,* ***a)*** *The difference between control group and I/R group was statistically significant (P 0.05),* ***b)*** *The difference between control group and MSC group was statistically significant (P<0.05),* ***c)*** *The difference between I/R group and MSC group was statistically significant (P<0.05)*

Table 2. *Hydroxyproline levels of the groups*

Groups	Hydroxyproline Levels		
	Min	Max	Median
Control group	211.39	1113.75	633.38[b]
I/R group	89.87	795.85	476.31[c]
MSC group	671.55	1453.17	1172.97[b,c]

*Values are presented as median (intrequartile range) *Kruskal-Wallis test,* ***a)*** *The difference between Control group and I/R group was statistically significant (P<0.05),* ***b)****The difference between Control group and MSC group was statistically significant (P<0.05),* ***c)*** *The difference between I/R group and MSC group was statistically significant (P<0.05)*

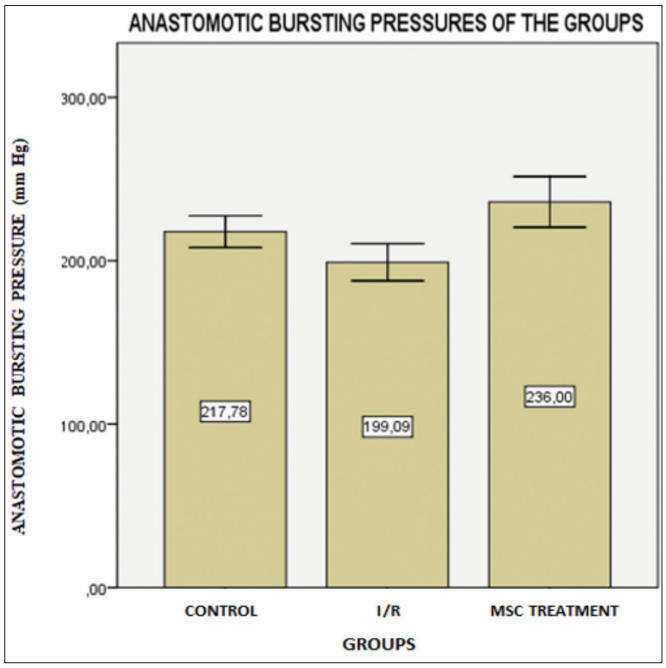

Fig 3. Anastomotic bursting pressure levels of the groups

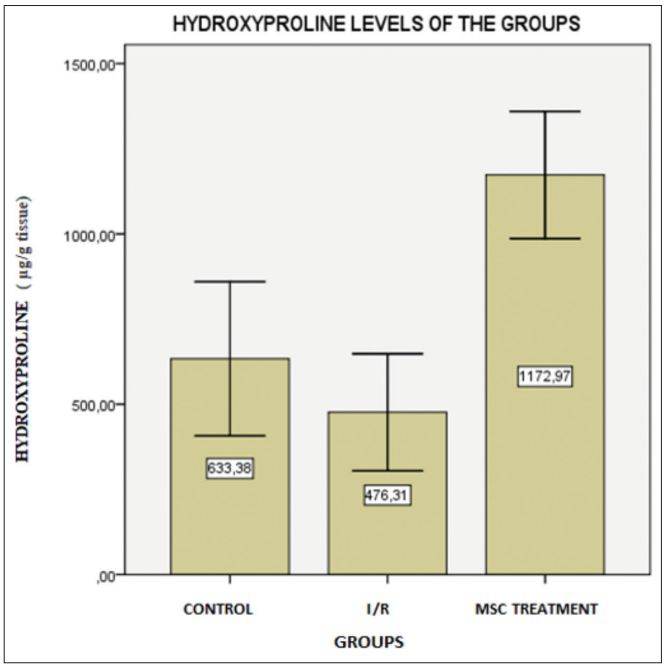

Fig 4. Hydroxyproline levels of the groups

additional surgical pathology. It was excluded from the experiment as it was thought to be caused by anesthesia. No pathology developed in the other rats. The experiment was completed successfully. A 5 cm intestinal segment, including the anastomosis area obtained with the last surgical procedure, was subjected to burst pressure measurements. Then the anastomosis site in the remaining tissue was resected and subjected to hydroxyproline measurements. The results were evaluated statistically. The results are detailed in *Table 1* and *Table 2*.

Mean anastomotic bursting pressure levels were measured as 217.78 mmHg in control group, 199.09 mmHg in I/R Injury group and 236 mmHg in MSC treatment group animals. Compared with control and MSC group animals, anastomotic bursting pressure levels of I/R injury group animals were found to be significantly low (P=0.041 and P<0.001 respectively). There was no significant difference between the control and MSC treatment groups in terms of anastomotic bursting pressure levels *(Fig. 3)*.

Mean hydroxyproline levels were measured as 633.38 in control group animals, 476.31 in I/R group animals and 1172.92 in MSC treatment group animals. There was no significant difference between the control and I/R injury group rats in terms of HP levels. The highest HP levels were noted in MSC group animals. Compared with control and I/R injury group rats, HP levels were found to be significantly high (P=0.002 and P<0.001 respectively) *(Fig. 4)*.

Discussion

Intestinal anastomoses are operations that are frequently performed in surgical clinics. Intestinal resection and anastomosis may be required for many reasons such as ileus, mesenteric ischemia, tumor, bleeding, diverticulum perforations, and stab wounds. In the clinical sense, intestinal structure, blood supply level, intra-abdominal contamination, surgical technique and age of the patient have an effect on anastomosis safety. Anastomotic leakage is

a pathology with high morbidity and mortality. Treatment procedures to maximize anastomosis safety have been tried over time. Academic studies for this purpose are frequently encountered in the literature. The most emphasized parameter in anastomosis safety is the vitality of the intestinal structure, that is, whether it is ischemic or not. It is a known fact that disruption of intestinal oxygenation will adversely affect healing [10]. However, the more effective damage is the destruction caused by the oxygen radicals of the reperfusion that develops after ischemia [1,11,12].

The release of many vasoactive mediators, cytokines and free oxygen radicals and leukocyte activation from reperfused intestines lead to endothelial dysfunction and edema. Reperfusion may lead to more severe injury than the injury results from ischemia itself. A variety of therapeutic agents have been studied in experimental studies to alleviate the adverse effects of I/R injury on the colonic anastomotic healing process. Commonly anti-inflammatory and/or antioxidant agents have been used to reduce ischemia or prevent ischemia-reperfusion injury [1,4,13].

When the experimental studies are examined, it is seen that mostly mechanical and biochemical parameters are used to evaluate the strength of the colonic anastomosis. Mechanically, the measurement of anastomotic bursting pressure is a commonly used method to examine the safety of colonic anastomosis in experimental studies. Christensen et al.[14] showed that bursting pressure is a meaningful parameter, since anastomotic disruption occurs at the maximum bursting pressure point. At such, the bursting pressure is a more accurate parameter to evaluate the bursting strength than the bursting wall tension [14,15]. In our study, we used the bursting pressure measurement method to evaluate the intestinal anastomoses between groups. We examined the significance levels between the data obtained in this way.

On the molecular level, one of the most meaningful parameters to examine anastomotic strength is tissue collagen content. Collagen fibers are the most important component of the wound healing process and primary responsible for the development of strength. Hydroxyproline is found only in collagen and elastin in animals. Therefore, the HP level in animals is a valuable measure in wound healing. On the fifth and seventh days after surgery, collagen synthesis reaches the peak and the wound strength is mainly due to these newly formed, organized collagen fibers [16,17]. In our experimental colon anastomosis model, we measured the HP level in tissue samples taken from the anastomosis line on the 7th day, when collagen synthesis is at its maximum. We compared the level of anastomosis robustness by looking at the statistical significance level of the results we obtained.

Mesenchymal stem cells (MSCs) are multipotent cells and easily differentiate into mesenchymal lineages. Currently MSCs are commonly preferred in the clinical treatment of various diseases due to biologic characteristic. Easy isolation and *in vitro* cultivation of these cells urge investigators to use them commonly. Particularly due to their high immunoregulatory capacity, MSCs are commonly used in diseases associated with immune system alterations. Adas et al.[18] showed that MSCs significantly accelerated the healing parameters for ischemic colonic anastomosis and increased the level of hydroxyproline on the seventh postoperative day. They also stressed that the histological parameters, necrosis and collagen deposition were also found to be important for the healing of ischemic colonic anastomosis. However, they also reported that MSCs did not accelerated angiogenesis in their study. Caziucet et al.[5] found that stem cells increase bursting pressure by elevating the rate of angiogenesis. Stem cells can be obtained from bone marrow or adipose tissue [19,20]. In our study, we used adipose tissue-derived stem cells which have the capability for direct differentiation to endothelial cells as well as indirectly angiogenic growth factor secretion [21,22].

When the literature is examined, we can see that many studies seek an answer to the question of what we can do for anastomosis safety. Similar to the experimental study we used, it was done by trying different substances. Sayin et al.[1] used montelukast and achieved significant results. Akarsu et al.[2] used simvastatin. Pehlivanlı et al.[4] used dexpanthenol or coenzyme Q10. It is seen that the substances used in the studies generally have anti-inflammatory and/or antioxidant properties. We think that the general structure of MSC will provide an effective improvement in the anastomosis line, since it has anti-inflammatory, antioxidant and angiogenic properties, as well as being multipotent and differentiable.

In our study, when the burst pressure measurements were examined, we found that the highest value was in the MSC group. Burst pressure values of the MSC group were significantly higher than the I/R group. When HP values, which are our other parameters, were examined, we found that the results obtained in the MSC group were significantly higher than both the control group and the I/R group. When the data obtained were examined, it was seen that MSC had positive effects on the healing of colon anastomosis.

In conclusion, our results showed that local application of MSCs improve the healing process of colonic anastomosis subjected to ischemia reperfusion injury. Both anastomotic bursting pressure and hydroxyproline levels considerably supported this finding. We think healing effects of MSCs on the wound healing of colonic anastomosis may be due to its anti-inflammatory, antioxidant and angiogenic effects. Of course, further investigations are needed for clinical topical usage of MSCs on colonic anastomosis subjected to an ischemic impact.

Statement of Author Contributions

H.Ö.: work management, article writing, experimental procedure follow-up; G.K.: design, article writing, literature review, statistics; H.B.: biochemical analysis; M.N.: stem cell production, experimental procedure follow-up; M.G.: design, article writing, literature review; Ç.E.D.: background assessment, review of results and final decision

Conflict of Interest

We declare that there is no conflict of interest.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 601-608, 2021
DOI: 10.9775/kvfd.2021.25952

RESEARCH ARTICLE

The Effects of Antifreeze Proteins I and III Supplemented Medium on Cryopreserved Rat Ovaries in Different Durations

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Article ID: KVFD-2021-25952 Received: 26.05.2021 Accepted: 16.09.2021 Published Online: 17.09.2021

Abstract

Cryopreservation techniques achieved by optimization of the composition and concentration of cryoprotectants not only reduce cryoinjury to ovarian tissue but also enhance survival. Anti-Freeze Proteins (AFPs) have been acknowledged to have positive effects on the survival of oocytes, embryos and ovaries during cryopreservation. AFPs drop the freezing point beneath the balance point of melting by binding ice crystals; restricting their expansion and recrystallization. This study aimed to investigate the protective effects of AFP I and III separately or in combination on the vitrification and warming procedures of ovarian tissues which were vitrified for one week and one month. Ovaries were obtained from rats and randomly assigned to four groups according to the AFP supplements in the media used during freeze/warm procedures: 1) control group (medium without AFP); 2) AFP I; 3) AFP III; 4) AFP I + AFP III groups. The groups were further organized according to the duration of vitrification, 1 week or 1 month. Ovaries were evaluated morphologically by a grading system, TUNEL assay and by transmission electron microscopy. AFP supplementation decreased apoptosis and follicular damage. Supplementation of AFP type I and type III has cryoprotective roles in ovaries which were vitrified for 1 week and 1 month. Additionally, AFP I+AFP III supplementation were shown to have more protection in long term.

Keywords: *Anti-freeze protein, Cryopreservation, Ovary, Vitrification, Rat*

Antifreeze Protein I ve III Eklenmiş Medyumun Farklı Sürelerde Kryoprezerve Edilmiş Sıçan Overi Üzerine Etkisi

Öz

Kriyoprotektanların kompozisyonu ve farklı konsantrasyonlarının eklenmesiyle geliştirilen kriyoprezervasyon teknikleri, over dokusunda sadece dondurma hasarını indirgemekle kalmaz, aynı zamanda over dokusunun devamını da sağlar. Anti-freeze Proteinlerin (AFP'ler) kriyoprezervasyon sırasında oositlerin, embriyoların ve yumurtalıkların hayatta kalması üzerinde olumlu etkileri olduğu kabul edilmiştir. AFP'ler, buz kristallerini bağlayarak donma noktasını erime noktasının altına düşürerek ve buz kristallerinin genişlemelerini ve yeniden oluşumunu sınırlandırır. Bu çalışmada, AFP I ve III'ün ayrı ayrı veya kombinasyon halinde hem bir hafta hemde bir ay süreyle vitrifiye edilen over dokularının çözdürme işleminden sonra koruyucu etkilerinin araştırılması amaçlandı. Sıçanlardan elde edilen overler vitrifikasyon/çözdürme prosedürleri sırasında kullanılan medyumdaki AFP takviyelerine göre rastgele dört gruba ayrıldı: 1) Kontrol grubu (AFP eklenmemiş medyum) 2) AFP I; 3) AFP III; 4) AFP I + AFP III grupları. Gruplar ayrıca vitrifikasyon süresine göre 1 hafta veya 1 ay olarak düzenlendi. Over dokularına; morfolojik olarak skorlama yapıldı. Ayrıca TUNEL metodu ile apoptozis açısından ve geçirimli electron mikroskobu ile ultrasütrüktürel açıdan değerlendirildi. AFP eklenmesi over dokularında apoptozisi ve foliküler hasarı azaltmıştır. AFP tip I ve tip III'ün eklenmesi, 1 hafta ve 1 ay süreyle vitrifiye edilen overlerde kriyo koruma sağlamıştır. Ek olarak, AFP I + AFP III takviyesinin uzun vadede daha fazla korumaya sahip olduğu gösterilmiştir.

Anahtar sözcükler: *Anti-freeze protein, Kriyokoruma, Over, Vitrifikasyon, Rat*

INTRODUCTION

Cryopreservation of oocytes, embryos and ovarian tissues, is a widely used technique particularly preferred for the preservation of fertility in cancer patients [1]. However, damages such as follicle loss, stromal cell destruction and programmed cell death could occur during the cryopreservation of ovarian tissues [2]. With recent advances

How to cite this article?

Erkanli Senturk G, Saygi HI: The effects of antifreeze proteins I and III Supplemented medium on cryopreserved rat ovaries in different durations. *Kafkas Univ Vet Fak Derg,* 27 (5): 601-608, 2021.
DOI: 10.9775/kvfd.2021.25952

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in cryopreservation techniques, cryo-injury rates were decreased and the survival duration of the tissues was increased [3-5].

The efficiency of cryopreservation depends on the composition and concentration of cryoprotectants in the cryopreservation medium [1]. Survival of oocytes can be improved by adding cryoprotectants to the cryopreservation medium [5].

Antifreeze proteins (AFPs) are a group of polypeptides found in the serum of Antarctic fish that can live under sub-zero temperatures which were first identified by DeVries and Wohlschlag in 1969 [6]. An animal studies showed that these proteins had positive effects on cryopreservation of sperm [7], mouse ovaries [1], mouse oocytes [8]. AFPs decrease freezing point beneath the balance point of melting by binding ice crystals. Therefore, they prevent expansion and recrystallization. Additionally, AFPs protect cell membranes from physical damage [9,10]. AFPs have also been shown to have beneficial effects on ovary vitrification [11]. Additionally, it was thought that they can be used to diminish cryo-injury during cryopreservation and increase survival via protection of follicular structure in the mouse ovary [12].

AFP type I is mostly composed of alanine with 4.3 and 3.3 kDa sized helical structures whereas AFP type II is cysteine rich and its helical structures are 14 kDa in size. On the other hand, AFP type III has only 66 amino acids and it lacks cysteine and alanine [6].

In the present study, we aimed to investigate the protective effects of AFP I and III supplemented cryopreservation medium on vitrified and warmed rat ovarian tissues at two different time points.

Material and Methods

Experimental Animals

Forty-eight Wistar albino female rats were housed with a 12-h-light/dark cycle at 22°C and fed *ad libitum* according to animal care guidelines. The ethical approval was obtained from Yeditepe University, Animal Care Committee with approved number 15.12.2014/435.

Vitrification and Warming of Rat Ovaries

Whole ovaries were obtained from rats after CO_2 inhalation. The ovaries were first randomly assigned into two groups according to the vitrification durations: 1) Ovaries that were warmed after vitrification of one week; 2) Ovaries that were warmed after vitrification for one month. Afterwards, for each of the vitrification durations, the ovaries were further assigned into four groups depending on the AFP supplements used in the vitrification and warming media: 1) Control group (vitrification medium without AFP); 2) AFP I supplemented medium (AFP I Group); 3) AFP III supplemented medium (AFP III Group); AFP I + AFP III supplemented medium (AFP I + AFP III Group).

The vitrification and warming media were supplemented with 10 mg/mL of type I AFP, 10 mg/mL of type III AFP, and the combination of both. The amount of AFP proteins was determined according to previously published data [11]. The chemical features of AFP proteins are shown in *Table 1*.

Ovaries were vitrified by a two-step process as previously described [10,11]. Initially, ovaries were equilibrated for 10 min at room temperature in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 20% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 7.5% dimethylsulfoxide and ethylene glycol (Sigma-Aldrich St. Louis, MO, USA). Afterwards, ovaries were incubated in the vitrification medium (D-PBS containing 20% FBS, 20% dimethylsulfoxide, 20% ethylene glycol, and 0.5 M sucrose) for 5 min at room temperature. Finally, the ovaries were placed into 1.5 mL cryovials (Nunc, Denmark) filled with liquid nitrogen and were stored for either 1 week or 1 month.

One week and 1 month after vitrification, ovaries were warmed as follows. Initially, the ovaries were brought out to air for 10 sec. Afterwards, they were incubated at room temperature by sequential 5-min equilibrations in 1, 0.5, 0.25, and 0 M sucrose (Sigma-Aldrich) solutions respectively. D-PBS with 20% FBS was used as the basal medium for both vitrification and warming solutions. For the AFP-supplemented groups, vitrification and warming media were supplemented with 10 mg/mL AFP type I and/or type III.

Histological Procedure

After the cryopreservation (7 or 30 days), all ovaries were fixed

Table 1. *The chemical features of AFP proteins*

Characteristic	AFP Type I	AFP Type III
Molecular Mass (kDa)	3.3-4.5	6.5
Primary Structure	alanine-rich multiple of eleven aa repeats	general
Secondary Structure	alpha helical amphiphilic	beta sandwich
Tertiary Structure	100% helix	not determined
Biosynthesis	prepro AFP	pro AFP
Protein Components	7	12
Natural Source	right-eyed flounders (winter flounder); sculpins (shorthorn)	ocean pout; wolffish

with 4% formaldehyde. Then, tissues were dehydrated and embedded in paraffin blocks. Five µm sections were taken from each paraffin block. Every fifth section was stained with Hematoxylin and Eosin and examined under light microscope (Leica DM 2500, Germany). The follicles were graded as tertiary, secondary, primary, or primordial follicles. Morphology of each follicle was evaluated according to the previously reported data [13,14] and presented in *Fig. 1* and *Fig. 2*. A total of 10 sections were evaluated for each animal. Percentages of follicles were compared statistically.

1- Primary and Primordial Follicles

Grade 1 (G1): Spherical shaped follicle with commensurate arrangement of granulosa cells,

Grade 2 (G2): Disarranged granulosa cells and a spherical oocyte in the follicle,

Grade 3 (G3): A spherical oocyte or vacuolation with pyknotic nuclei, abnormal granulosa cells

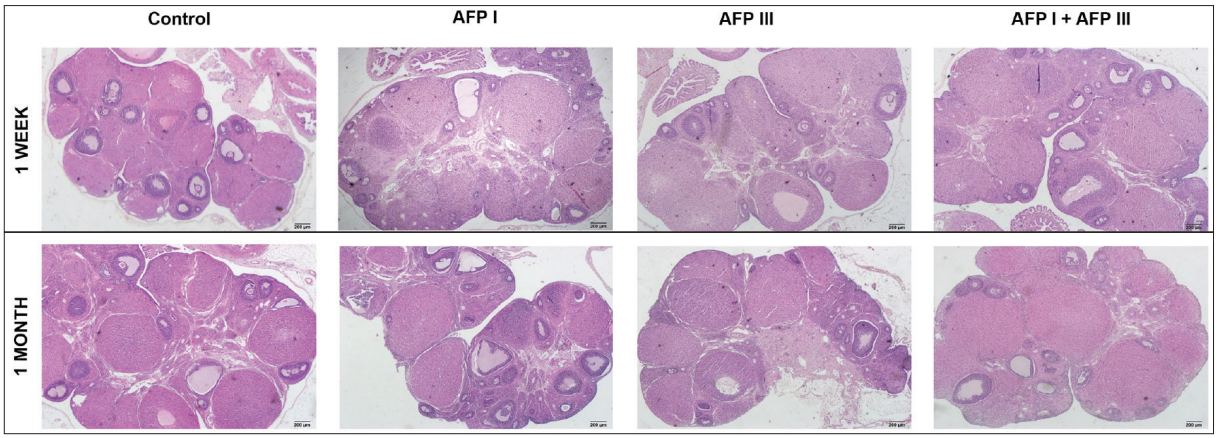

Fig 1. Morphology of rat ovaries in vitrification control, AFP I, AFP III, and AFP I+III groups after 1 week and 1 month. Hematoxylin and Eosin staining. Bar: 200 µm

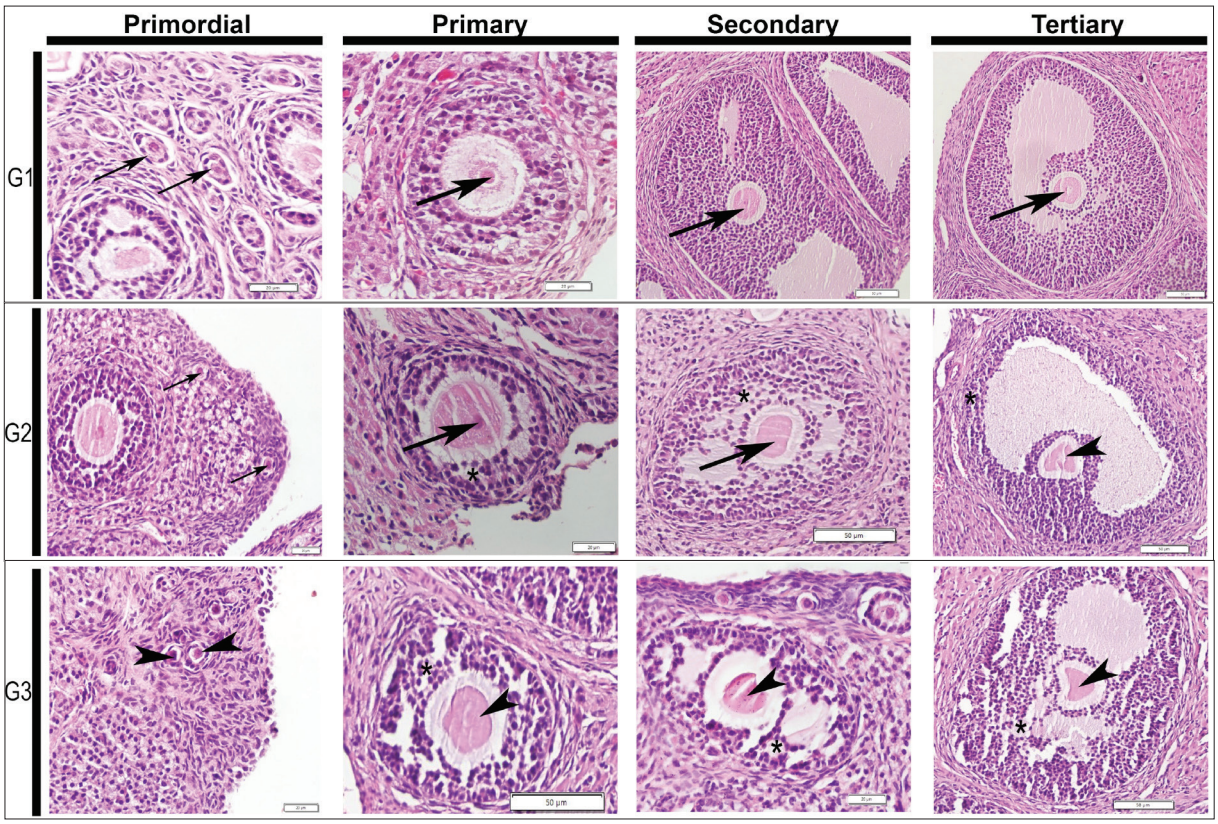

Fig 2. Morphological differences in rat ovarian follicles according to their grading scores. Spherical oocyte in primordial follicle *(thin arrow);* spherical oocyte *(thick arrow)* in primary, secondary and tertiary follicle; abnormal oocyte *(arrow head);* disarranged or abnormal granulosa cells (*). Hematoxylin and Eosin staining

2- Tertiary and Secondary Follicles

Grade 1 (G1): Intact spherical shaped follicle with commensurate arrangement of theca and granulosa cells and aspherical oocyte,

Grade 2 (G2): Intact morphology of theca cells, abnormal granulosa cells and a spherical oocyte,

Grade 3 (G3): Loss of or abnormal theca and granulosa cells without an oocyte or abnormal oocyte in the follicle.

TUNEL Assay for Apoptosis

Five µm sections from all groups were placed on positively charged slides for TUNEL assay. TUNEL assay was utilized according to the manufacturer's instructions (Biotium, CF™ 488A TUNEL Assay Apoptosis Detection Kit). Sections were incubated with proteinase K (5 min), washed with distilled water and incubated with 3% hydrogen peroxide in PBS (5 min). Then the slides were washed with PBS, put in the equilibrium buffer (30 min) and incubated in recombinant terminal transferase TdT enzyme (at 37°C for 1 h). The sections were then washed in buffer with agitation (15 s), followed by a wash in PBS and incubated with anti-digoxigenin conjugate (30 min). After washing in PBS, the sections were incubated with peroxidase solution for 6 min. After a final wash in distilled water, the slides were covered with Entellan (Merck, Darmstadt, Germany). Five randomly selected areas from each section were observed under fluorescence microscope (Leica DM 2500, Leica MC 170, Germany) and TUNEL positive cells were counted. Average values were evaluated.

Transmission Electron Microscopy

Vitrified and warmed ovaries from all groups were fixed with 2.5% glutaraldehyde in PBS (pH=7.4) and then incubated with osmium tetroxide for 1 h. All tissues were dehydrated in ascending ethanol series and then incubated in propylene oxide and embedded into Epoxy epon blocks. Semi-thin sections were taken from epon blocks by an ultramicrotome (Reichert, Germany) and stained with toluidine blue. Thin sections were contrasted with uranyl acetate and lead citrate and examined under transmission electron microscope (Jeol 1011, Japan, Olympus-Veleta TEM camera, Tokyo, Japan).

Statistical Analysis

Sample size is determined by power analysis. The result of the calculation was determined as n=8 for histological examinations. All calculations were calculated as type I error $P<0.05$, type II error (power) 0.20.

All results were compared with Mann-Whitney U or ANOVA test. Tukey's test was used for post-hoc test. Data were analyzed by Graphpad 3.0 prism. $P<0.05$ is considered as statistically significant.

Results

Morphological Evaluation

All ovaries were examined morphologically *(Fig. 1)* and follicles in ovaries were graded *(Fig. 2)*. In the control group (without supplementation of AFP), follicles in ovaries were significantly damaged after 1 week of vitrification. The damage became severe after 1 month of vitrification. Vitrification and warming caused granulosa cell degeneration and shrinkage of oocytes in the vitrification control group and there were less G1 follicles when compared to AFP supplemented groups ($P<0.01$, *Fig. 3*). Ovaries of AFP I and AFP III groups were less damaged when compared to the control group. There was less degeneration in granulosa cells and oocytes. Additionally, there were more numbers of G1 follicles when compared to the time-matched control group. Ovarian damage was found to be the highest in the control group after 1 month of vitrification ($P<0.05$, *Fig. 3*).

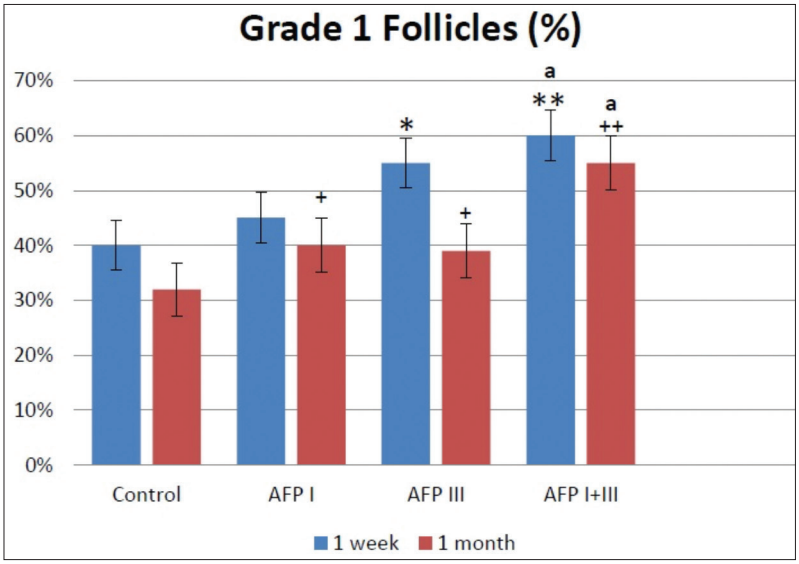

Fig 3. Percentage of all grade 1 follicles in vitrification control, AFP I, AFP III and AFP I + AFP III groups after 1 week and 1 month vitrified and warmed ovaries. * $P<0.05$, ** $P<0.01$ compared with time-matched vitrification control group, +: $P<0.05$, ++: $P<0.01$ compared with time-matched vitrification control group; a: $P<0.05$, compared with time-matched AFP I and AFP III

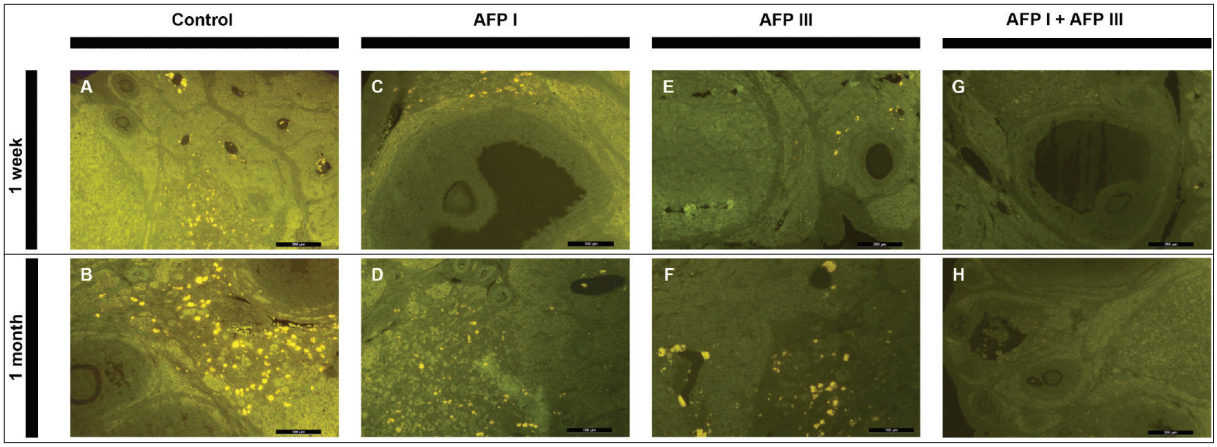

Fig 4. Apoptotic cells in ovarian follicles. **A** and **B)** Vitrification control group; **C** and **D)** AFP I; **E** and **F)** AFP III; **G** and **H)** AFP I + AFP III groups. TUNEL assay. A, E, G, H Bar: 200 µm, B, C, D, F Bar: 100 µm

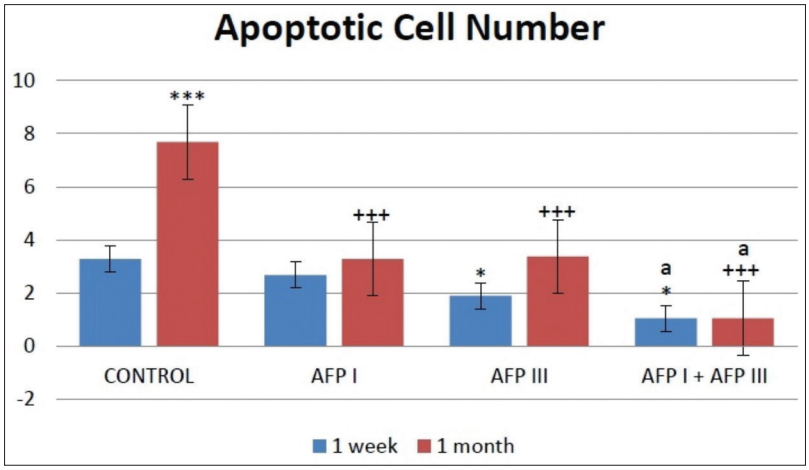

Fig 5. The graph of apoptotic cell numbers in vitrification control, AFP I, AFP III and AFP I + AFP III groups after 1 week and 1 month vitrified and warmed ovaries. * P<0.05 compared with time-matched vitrification control group, *** P<0.001 compared with 1week vitrification control group, +++: P<0.001 compared with time-matched vitrification control group, a: P<0.05 compared with time-matched AFP I and AFP III groups

The morphology of the ovaries of AFP I + AFP III groups were more intact. There was no or very minimum damage in granulosa cells and oocytes. Percentage of G1 follicles were the highest in AFP I + AFP III group when compared to the other groups (P<0.01, *Fig. 3)* in both durations. The protective effects of AFP I and AFP III on ovarian tissue, was higher when used in combination in both of the vitrification durations. Moreover, this protective effect was higher when compared to the control, AFP I and AFP III groups. Percentage of G1 follicles *(Fig. 3)* were higher in AFP III group when compared to AFP I group after 1 week of vitrification. Morphological differences in groups are shown in *Fig. 1* and *Fig. 2*.

Evaluation of Apoptosis

Apoptotic granulosa cell numbers decreased after 1 week of vitrification in AFP I+III group compared to vitrification control and AFP I and AFP III alone groups (P<0.05, *Fig. 4* and *Fig. 5)*. Furthermore, the apoptotic cell numbers were higher in the control group which had been vitrified for 1 month compared to the control group which had been vitrified for 1 week (P<0.001). AFP supplementation decreased apoptosis in both of the vitrification durations. Nevertheless, the decrease in apoptotic cell numbers was more prominent in AFP I + III groups in both of the vitrification durations when compared to the time-matched control groups *(Fig. 5)*. There was a significant decrease in apoptotic cell numbers particularly in the AFP I + AFP III group in which ovaries were vitrified for 1 month (P<0.001, *Fig. 5)*. The apoptotic cell numbers in this group were almost as close to the numbers in the AFP I + AFP III group in which ovaries were vitrified for 1 week. Apoptotic cell numbers were decreased in AFP I and AFP III alone groups underwent 1 month of vitrification when compared to time-matched vitrification control group (P<0.001, *Fig. 5)*.

Electron Microscopic Findings

The cellular organelles were damaged in the vitrification control group. There were more round mitochondria and the cell membranes of granulosa cells were disrupted with big vacuoles. The zona pellucida was abnormal and the area between the oocyte and follicular cells was increased. These findings were reversed in AFP groups. The zona pellucida between oocytes and cellular junctions among granulosa cells appeared normal in both AFP I and AFP III groups at both of the vitrification durations, whereas

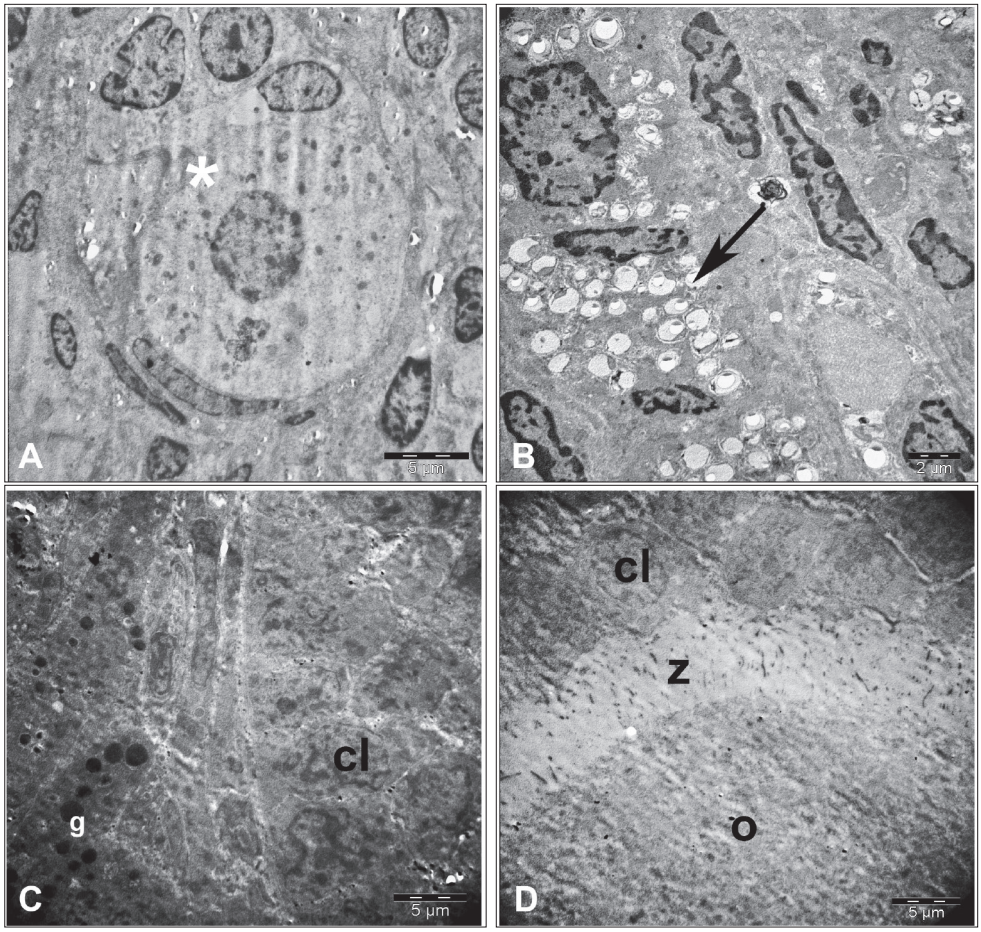

Fig 6. A, B) Vacuoles in granulosa cells (*) and damaged oocyte *(arrow)* in vitrification control group; **C, D)** less damages in granulosa (g) and cumulus cells (cl) and normal zona pellucida (z) and oolemma (o) in AFP I + AFP III group. Transmission electron micrographs. A, C, D Bar: 0.5 µm, B Bar: 0.2 µm

the cellular organelles and cell membranes of granulosa cells were slightly damaged. Moreover, the mitochondria were elongated and the size and amount of vacuoles were decreased in both AFP I and AFP III groups *(Fig. 6)*.

Discussion

In the present study, the cryoprotective effects of AFP proteins were evaluated in ovarian tissues which were vitrified for 1 week and 1 month. We observed that when AFP I and AFP III were added to the vitrification and warming media either separately or in combination, they exerted protective effects at the tissue, cellular and sub-cellular levels.

The combined supplementation of AFP I and AFP III to the vitrification medium and the warming medium, protects vitrified (1 week and 1 month) and warmed ovaries when compared to AFP supplementation alone.

AFPs can act in cryoprotection through two mechanisms. The first one is by inhibiting recrystallization, ice growth and nucleation of ice. The second one is by protecting the cell membrane and reducing cytotoxicity during cooling. AFPs can also inhibit recrystallization and ice nucleation at the warming stage [15]. Moreover, it was shown that the same AFP concentrations that we used in our study for vitrification and warming procedures protected frozen and warmed whole ovaries [1,15]. Based on this knowledge, in this study, we added AFPs into both vitrification and warming media and examined the protective effects of this AFP supplemented media on the structure of vitrified and warmed ovarian tissue at the light microscopic and the ultrastructural levels. We demonstrated that adding AFPs in vitrification and warming media has beneficial effects on the cryoprotection of whole ovaries in terms of both oocytes and granulosa cells.

Most studies showed that higher concentrations of AFP have degenerative effects on cells and tissues [16]. Other studies emphasized that lower concentrations of AFP have beneficial effects by regulating the survival of oocytes, whereas higher concentrations decreased the survival rates [12]. Moreover, Lee et al.[12] explained that a concentration of 20 mg/mL of AFP had better effects than 5 mg/mL of AFP. A synergistic effect that may have counterbalanced each other is caused by higher concentration of AFPs which are a mixture of AFP type I and III. As shown by previous studies, high concentrations of cryoprotectants cause osmotic stress and damage in follicles of cryopreserved mouse ovaries [16].

Furthermore, AFP supplemented cryopreservation media improved the integrity of follicles in vitrified and warmed

ovaries. Additionally, our results demonstrated that AFP I and AFP III when combined had better effects on vitrified and warmed ovaries even in long term vitrifications.

In our present study, we vitrified ovaries for one week and one month. During vitrification, ovaries may be damaged due to ice crystal formation and/or recrystallization. AFP I and AFP III supplementation to the vitrification and warming media also had beneficial effects at longer vitrification times. AFP I and AFP III, when supplemented together had more protective effects at longer vitrification durations when compared to AFP I or AFP III supplemented alone.

Two different AFP types were used at the same concentration instead of using only one type of the AFPs. And it was shown that the cryoprotectant solution supplemented with two equal concentrations of two different AFPs is more protective than only one type of AFP.

AFPs protect the membrane integrity in mouse oocytes [9,15]. Follicular integrity of ovaries after vitrification with AFP supplemented media is better than that of AFP lacking media [1]. Consistent with our study, it was shown that vitrification solutions have beneficial ultrastructural effects on cellular membrane and organelles as well as on the protection of mitochondria [17]. The protection of cellular organelles, cell membrane and mitochondria after vitrification is very crucial for normal physiological function. AFP supplemented vitrification medium provides protection of primary oocytes and granulosa cells in follicles ultrastructurally.

In conclusion, to the best of our knowledge, our work is unique in describing the cryoprotective effects of AFP type I and III, and the combination of these proteins on the ovaries that were vitrified for different durations. We demonstrated that supplementation of the vitrification and warming media with AFP I and AFP III, and the combination of the two, had beneficial effects on follicle integrity, enhancement in whole ovaries, as well as in primordial follicles with primary oocytes, specifically after 1 week and 1 month vitrification. This study shows the AFPs' cryoprotective effects in long term and the comparison between different durations of vitrification. Further studies are required to clarify the exact mechanisms of how AFPs prevent cryo-injury.

Acknowledgements

Authors thank to Assoc. Prof. Dr. Yasemin Seval for her probono editing of this manuscript and Sümeyye Bayrak, Selim Can Berk and Mehmet Alp Gunduz for help in laboratory processes.

Conflict of Interest

Authors declare that they have no conflict of interest.

Author Contributions

Gozde Erkanli Senturk: Hypothesis, manuscript writing, animal procedure and histological procedures, statistics; Halil Ibrahim Saygı: Transmission electron microscopic procedure and evaluation, statistics.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available within the article.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 609-616, 2021
DOI: 10.9775/kvfd.2021.25965

RESEARCH ARTICLE

Haematological Profile After Use of Titanium Double Shanked Ligation Clips in Laparoscopic Appendectomy: An Experimental Study on Rat Model

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Article ID: KVFD-2021-25965 Received: 01.05.2021 Accepted: 07.08.2021 Published Online: 07.08.2021

Abstract

The aim of the study was to determine possible adverse effects on haematological parameters of the rats, using different surgical techniques, i.e. titanium double shanked ligation (DS) clips and endoloop polyglactin 910 suture material in laparoscopic appendectomy. The use of DS clips and other similar methods is not widely represented in veterinary surgery, as is the case in humans. Fifty rats were randomized into three groups: group 1 in which the appendiceal base was closed with a endoloop polyglactin 910 ligatures, 20 rats, group 2 in which the appendectomy was closed with DS titanium clips, 20 rats. Group 3 was the control, in which the animals were not subjected to the surgical procedure, 10 rats. Blood was drawn from the caudal vein of ten animals from each experimental group, 7 and 28 days post-surgery. Thrombocytopenia was found in the DS clips group, and hypochromia with hypochromic erythrocytes, as well as neutrophilia and lymphopenia in both experimental groups. From a haematological standpoint, DS clips are more applicable for potential patients than endoloop polyglactin 910 ligatures.

Keywords: *Comparison with suture material, Hypochromic anaemia, Lymphopenia, Neutrophilia, Rat*

Laparoskopik Apendektomide Titanyum Çift Uçlu Ligasyon Klipslerinin Kullanımı Sonrası Hematolojik Profil: Ratlar Modelinde Deneysel Bir Çalışma

Öz

Bu çalışmada, ratlarda laparoskopik apendektomide titanyum çift uçlu ligasyon (DS) klipsleri ve endoloop poliglaktin 910 sütür materyali gibi farklı cerrahi teknikler kullanılarak hematolojik parametreler üzerine olası olumsuz etkilerinin belirlenmesi amaçlanmıştır. DS klipslerinin ve diğer benzer yöntemlerin kullanımı, insanlarda olduğu gibi veteriner cerrahisinde yaygın kullanım alanı bulmamaktadır. Elli rat rastgele üç gruba ayrılmış: Grup 1'de (n=20 rat), apendiks tabanının bir endoloop poliglaktin 910 ligatür ile kapatıldığı apendektomi, Grup 2'de (n=20 rat) DS titanyum klipslerle kapatıldığı apendektomi uygulanmıştır. Grup 3 (n=10 rat), ratların cerrahi işleme tabi tutulmadığı kontrol grubunu oluşturmuştur. Cerrahi işlemden 7 ile 28 gün sonra her deney grubundan on hayvanın kaudal damarlarından kan alınmış. DS klips uygulanan grupta trombositopeni ve her iki deney grubunda hipokromik eritrositlerle birlikte hipokromi ve ayrıca nötrofili ve lenfopeni saptanmış. Hematolojik açıdan, DS klipsleri potansiyel hastalar için endoloop poliglaktin 910 ligatürlerinden daha uygundur.

Anahtar sözcükler: *Sutur materyali ile karşılaştırma, Hipokromik anemi, Lenfopeni, Nötrofili, Rat*

How to cite this article?

Bajrić A, Katica M, Smajović A, Katica A, Sivić M, Rukavina D, Zahirović A, Bajrić A, Čelebičić M, Hassan Ahmed N, Delibegović S: Haematological profile after use of titanium double shanked ligation clips in laparoscopic appendectomy: An experimental study on rat model. *Kafkas Univ Vet Fak Derg,* 27 (5): 609-616, 2021. DOI: 10.9775/kvfd.2021.25965

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Introduction

Resection of the appendix may be performed routinely using several well-known techniques. One of the standard and cheap technique used is endoloop polyglactin 910 ligatures [1]. A more expensive technique is the use of staplers with titanium clips, which has advantages in laparoscopic surgery [2,3] as a possible alternative when other techniques are not able to close the appendiceal stump [4,5]. Using non-resorptive Hem-o-lok plastic clips is also another alternative method which has some advantages [1,6]. The effect of each of these techniques on haematological parameters has already been described [1,3].

Titanium DS clips are made of the same material as stapler clips, but they are constructed differently [7,8]. Their effect on haematological parameters after the application is unknown. Since DS clips and stapler clips are made of the same material, it may be presumed that their effect on the haematological profile will be similar. Any possibly undesirable effect of DS clips on haematological parameters may have implications in their clinical use in the end.

In general, monitoring the complete haematological status of patients is an extremly important aspect during postoperative care, through which is possible faster prevention of unwanted potential health disorders [3].

For this reason, we conducted an experimental study using rodents, in order to establish any undesirable effect of DS clips on haematological parameters and compare them with endoloop polyglactin 910 ligatures, following laparoscopic appendectomy.

Material and Methods

Ethical Statement

The research study was compatible with the Law on Animal Welfare of Bosnia and Herzegovina for experiments involving animals, and the Ethics Committee of the Veterinary Faculty of the University of Sarajevo issued a positive opinion about it, number 01-02-18-16/19.

Animals

Wistar rats were used in the study, aged 12-14 weeks and weighing between 200 and 300 g. The animals were housed in a vivarium with a natural light-dark cycle, with appropriate environmental temperature and humidity. They were given pellet feed for rodents and water *ad libitum*. Food was removed 24 h before the surgical procedures. Fifty rats were randomized into three groups: Group 1 (n=20 rats) in which the appendiceal base was closed with an endoloop polyglactin 910 (Vicryl®, ETHICON) ligatures, Group 2 (n=20 rats) in which the appendectomy was closed with DS titanium clips. In Group 3 with 10 rats, actually the control and the animals were not subjected to any surgical procedures.

Surgical Procedure

The rats were anaesthetized using the combination of 10 mg/kg xylazine HCl (Rompun® 2%, Bayer) and Ketamine HCl 50 mg/kg (International B.V. Boxmeer, The Netherlands), intramuscularly. The fur covering of the abdomen was shaved, and the skin was disinfected with a povidone-iodine solution. They were laid on the operation table on their backs. Their extremities were fixed with sticky tape. An incision was made along the medial line. The large caecum sac was located in the lower third of the abdominal cavity. The large caecum sac in rats has a closed end and is equivalent to the appendix in humans. That part was resected.

Laparotomy and skin closure were performed with a 3-0 continuous suture. No antibiotic therapy was used during or after the experiment. Throughout the observation period, all animals were monitored and subjected to clinical trials [1].

Parameters of Monitoring

Blood was drawn from the caudal vein of ten animals from each experimental group, 7- and 28-days post-surgery. Rats in the control group (n=10) also had blood drawn and it was used for comparison.

Haematological Tests

An "Idexx Laser Cyte" flow haemocytometer was used, and the following parameters were analysed: Erythrocytes (count) (RBC) (10^{12}/L) and Leukocytes (count) (WBC) (10^{9}/L), Platelets (count) (PLT), Haemoglobin (HGB) (g/dL), Haematocrits (HCT) (%), Mean Corpuscular Volume (MCV) (fl), Mean Corpuscular Haemoglobin (MCH) (pg) and Mean Corpuscular Haemoglobin Concentration (MCHC) (g /dL).

Microscopic analysis of poikilocytic forms of erythrocytes involved microscope examination of blood smears, previously stained by the Giemsa method. Poikilocytosis was classified semi-quantitatively according to similar research [9,10], using the following criteria: absent (0%), rare (0.05% - 0.5%), mild (>0.5% - 3%), moderate (>3% - 10%) or marked (>10%).

The number and type of poikilocytic forms of erythrocytes were recorded as the percentage of erythrocytes. Within each stained blood smear from the test animals, 1000 erythrocytes were counted and analysed using a binocular light microscope, Motic Type 102 M, with 900 x magnification [1,3].

The relative values of the leukogram for lymphocytes (L), neutrophils (N), monocytes (M), basophils (B) and acidophiles (A) are shown as percentages (%), after microscopic differentiation of 1000 of them within each blood smear

from the test animals, from the experimental and the control group, using a Motic Type102 M binocular light microscope with 900 x magnification [1].

Statistical Analysis

Statistical analysis of data was performed using IBM SPSS Statistics for Windows, Version 24. The data were processed using a parameter (ANOVA) test or a non-parameter test (Kruskal Wallis) depending on whether the data were normally distributed or not. The Shapiro-Wilk test was used to test normality, and $P<0.05$ was taken as statistically significance. If the result showed statistical significance, post hoc tests were conducted (Tuckey, Dunett) to establish between which groups that difference existed [11].

Results

Fig. 1-8 show the mean values and standard deviations of RBC, HCT, HGB, MCV, MCH, MCHC, WBC and PLT, respectively. The highest values of RBC, HCT and WBC were noticed in the Polyglactin 910 group on day 28, whilst the MCHC and PLT values were the highest in the Polyglactin 910 group on day 7. What is extremely interesting is that the values of HGB, MCV and MCH were lower in the test groups than in the control. The standard deviations calculated for all the test groups and for all parameters were quite large, which indicates the large dispersion of results in relation to the mean values calculated.

Fig. 9, which shows the mean cell values in the leucogram, clearly shows that lymphocytes (L) and neutrophils (N) dominate the total number in terms of percentage. Although in all the test groups the L values was above 55%, its highest value was in the control group (82.56%) and its lowest in the Polyglactin 910 group on day 7 (56.7%). In contrast to the situation, we can see with L, the presence

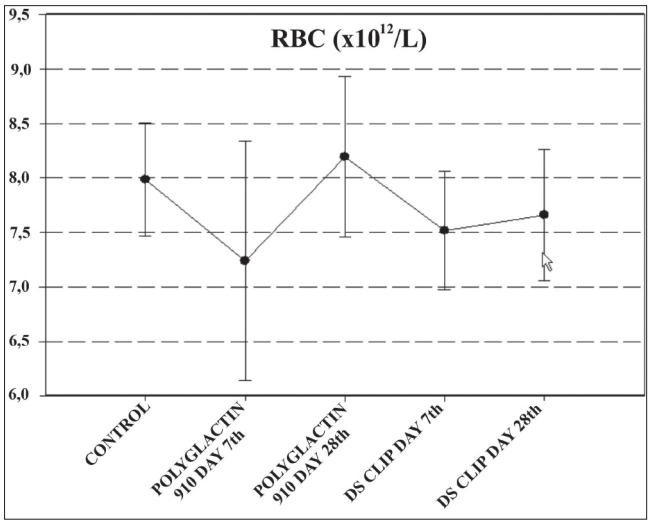

Fig 1. Changes in the number of erythrocytes (RBC) (mean value ± standard deviation)

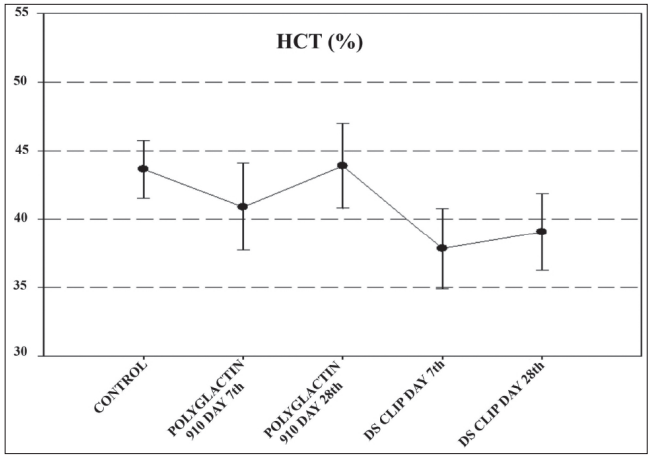

Fig 2. Changes in the haematocrit values obtained (HTC) (mean value ± standard deviation)

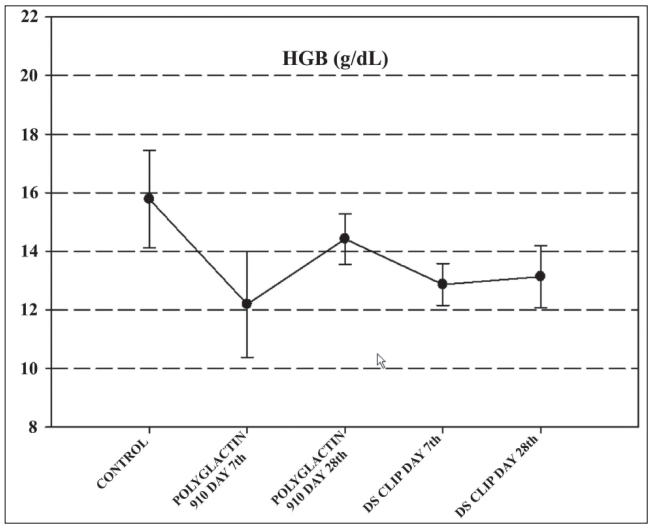

Fig 3. Changes in the haemoglobin values obtained (HGB) (mean value ± standard deviation)

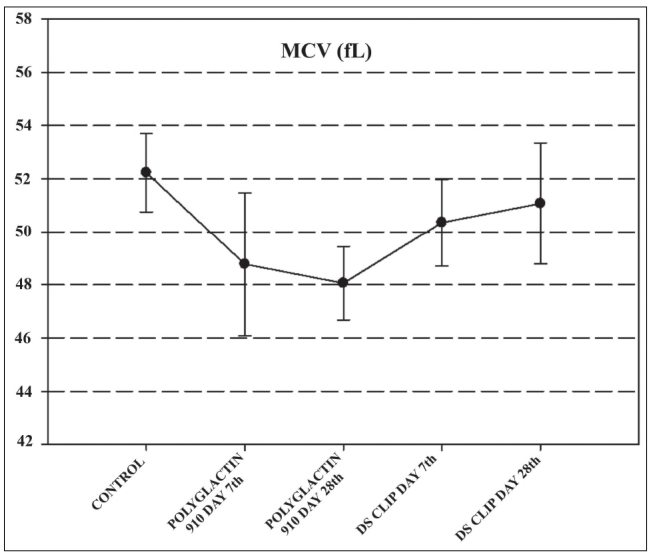

Fig 4. Changes in the MCV values obtained (mean value ± standard deviation)

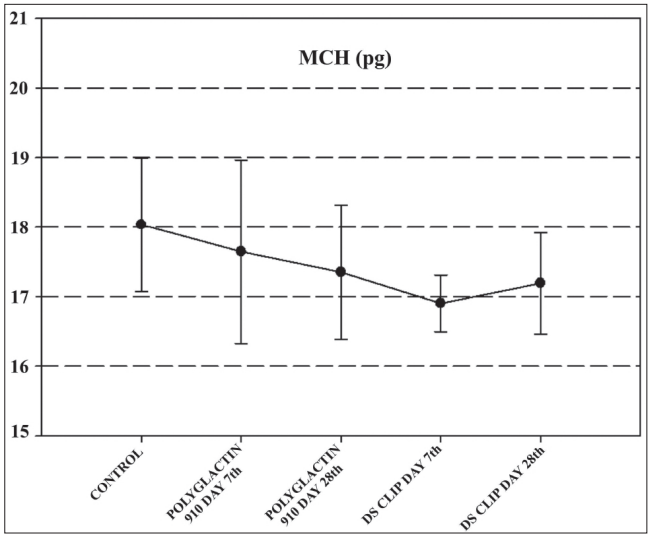

Fig 5. Changes in the values obtained (MCH) (mean value ± standard deviation)

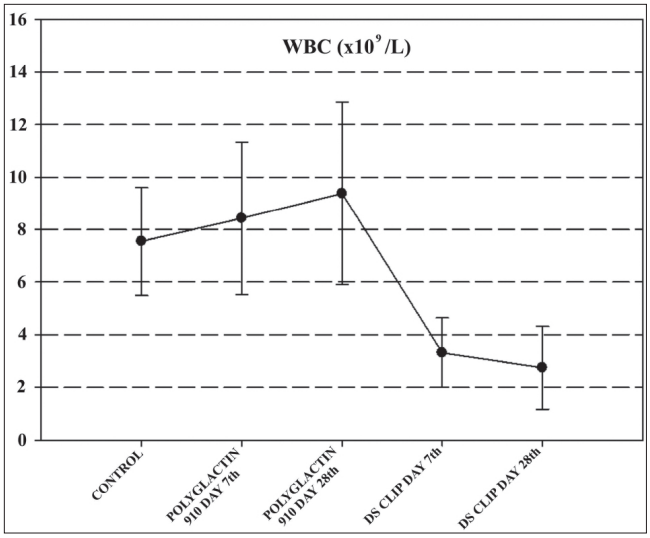

Fig 7. Changes in the haemoglobin leukocyte values obtained (WBC) (mean value ± standard deviation)

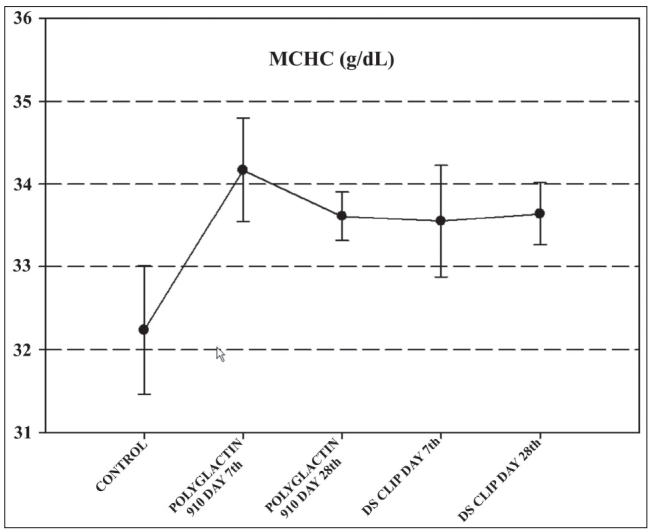

Fig 6. Changes in the values obtained (MCHC) (mean value ± standard deviation)

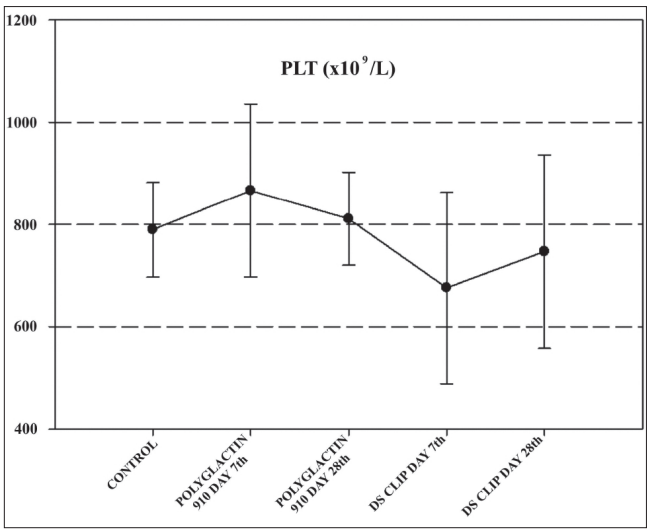

Fig 8. Changes in the thrombocyte values obtained (PTL) (mean value ± standard deviation)

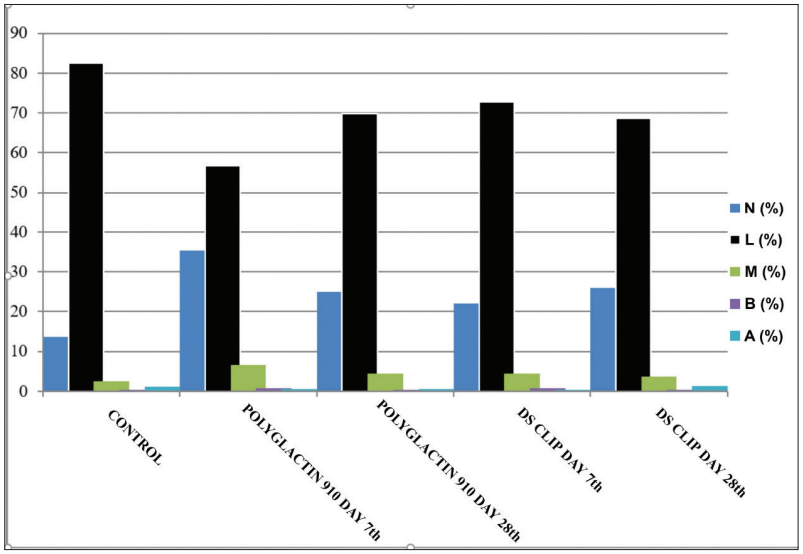

Fig 9. Leucogram (%) for the control and two experimental groups

Table 1. *The type of poikilocytic forms in peripheral blood smears in the control and experimental groups. The values represent the percentage (%) of poikilocytic forms in 1000 erythrocytes analysed*

Poikilocytic Forms of Erythrocytes	Control	Polyglactin 910 Day 7	Polyglactin 910 Day 28	DS Clip Day 7	DS Clip Day 28
OVALOCYTES	0.195	0.49	0.49	1.09	1.37
DACRIOCYTES	0.16	1.11	0.54	0.685	1.285
ANULOCYTES	1.025	3.45	4.56	2.435	11.105
ECHINOCYTES	0.495	1.09	1.46	0.17	1.37
STOMATOCYTES	0.27	0.50	1.77	1.11	0.155
DREPANOCYTES	0	0.02	0.00	0.07	0.08
SCHIZOCYTES	0.035	0.14	0.08	0.115	0.075
TARGET CELLS	0	3.74	0.82	0.44	1.405
ACANTOCYTES	0	3.03	0.00	0	1.015
SPHEROCYTES	0.01	2.56	1.57	0.98	2.335
RETICULOCYTES	0.24	0.64	0.12	0.64	0.12

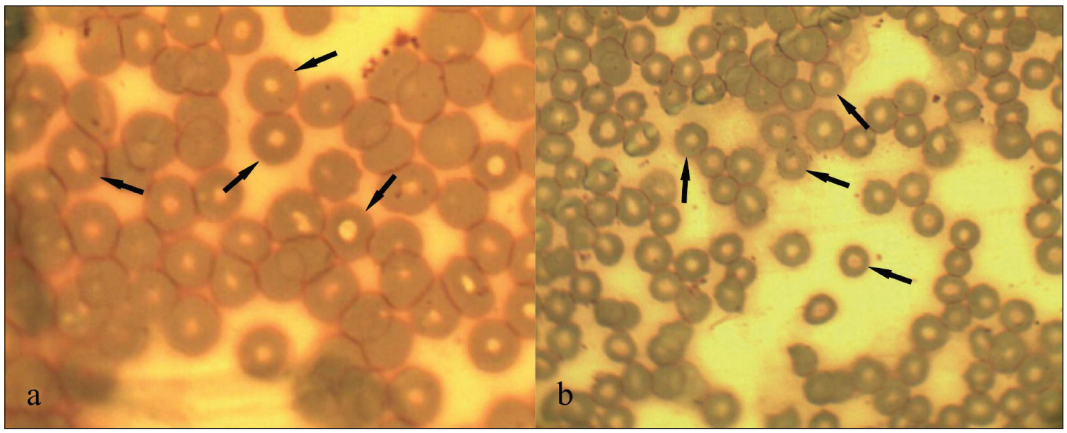

Fig 10. Hypochromic erythrocytes (anulocytes), Polyglactin 910 day 28 *(black arrow)* (a), and also hypochromic erythrocytes (anulocytes), DS clips day 28 *(black arrow)* (b)

Table 2. *Statistical analysis of the results obtained. The calculated values are the result of post hoc analysis after the ANOVA test disproved the null hypothesis*

Parameter	P -Value (ANOVA/ Kruskal Wallis)	P-Value (post hoc)									
		Polyglactin 910 Day 7-DS Clip Day 7	Polyglactin 910 Day 7-DS Clip Day 28	Polyglactin 910 Day 7-Control	Polyglactin 910 Day 7-910 Day 28	DS Clip Day 7-DS Clip Day 28	DS Clip Day 7-Control	DS Clip Day 7-Polyglactin 910 Day 28	DS Clip Day 28-Control	DS Clip Day 28-Polyglactin 910 Day 28	Control-Polyglactin 910 Day 28
RBC (x10^{12}/L)	0.012	1.0	1.0	0.593	0.024	1.0	0.9	0.045	1.0	0.29	1.0
HCT (%)	0.000	0.774	1.0	0.824	0.684	1.0	0.004	0.002	0.037	0.025	0.684
HGB (g/dL)	0.000	1.0	1.0	0.000	0.043	1.0	0.000	0.037	0.006	0.385	1.0
MCV (fL)	0.001	1.0	0.37	0.045	1.0	1.0	0.785	0.329	1.0	0.02	0.001
MCH (pg)	0.118	-	-	-	-	-	-	-	-	-	-
MCHC (g/dL)	0.000	0.153	0.284	0.000	0.234	0.997	0.000	0.999	0.000	1.0	0.000
WBC (x10^{9}/L)	0.000	0.000	0.000	0.936	0.909	0.983	0.004	0.000	0.001	0.000	0.469
PLT (x10^{9}/L)	0.085	-	-	-	-	-	-	-	-	-	-
N (%)	0.001	0.034	0.237	0.000	0.156	0.897	0.365	0.962	0.032	0.999	0.111
L (%)	0.000	0.01	0.092	0.000	0.051	0.902	0.264	0.970	0.044	0.999	0.079
M (%)	0.143	-	-	-	-	-	-	-	-	-	-
B (%)	0.321	-	-	-	-	-	-	-	-	-	-
A (%)	0.071	-	-	-	-	-	-	-	-	-	-

A statistically significant result is P<0.05

of N and monocytes (M) is highest in the Polyglactin 910 group on day 7 (35.4%; 6.6%, respectively), and lowest in the control group (13.56%; 2.56%, respectively). The values of basophils (B) and acidophiles (A) were without major deviations in all the groups.

In all groups, poikilocytic forms were present *(Table 1)*, with the marked presence of anulocytosis in the DS clip experimental group on day 28 (11.105%) *(Fig. 10-b)* and moderate anulocytosis in the Polyglactin 910 group on day 28 (4.56%) *(Fig. 10-a)*, and in the Polyglactin 910 group on day 7 (3.45%). A moderate presence of target cells was noticed in the Polyglactin 910 group on day 7 (3.74%). All the other poikilocytotic forms were mildly expressed (0.05-3%) within all the experimental groups. A complete absence of drepanocytes was noticed in the control group and the Polyglactin 910 group on day 28, of target cells in the control group, of acantocytes in the control and Polyglactin 910 groups on day 28, and in the DS clip group on day 7.

Multivariant analysis of data ($P<0.05$) showed a statistically significant difference between the groups for all parameters except MCH and PLT *(Table 2)*. Also, the values of monocytes, basophils, and eosinophils did not differ significantly between the groups. Post hoc analysis showed between which groups there was a statistically significant difference, as presented below. A statistically significant difference in RBC values was observed in the Polyglactin 910 group on day 7 and day 28, as well as between the DS clip group on day 7 and the Polyglactin 910 group on day 28. A statistically significant difference in HCT values was observed between the control group and the DS clip group on day 7 and the DS clip group on day 28. There was also a difference between the Polyglactin 910 group on day 28 and the DS clip group on day 7 and DS clip on day 28.

A difference in HGB values was observed between the control group and the Polyglactin 910 group on day 7, the DS clip group on day 7, and the DS clip group on day 28. There was also a statistically significant difference between the Polyglactin 910 group on day 28 and the Polyglactin 910 and DS clip groups on day 7.

A statistically significant difference in MCV values was observed between the control group and the Polyglactin 910 group on day 7 and the Polyglactin 910 group on day 28, as well as between the DS clip group on day 28 and the Polyglactin 910 group on day 28.

There was a statistically significant difference in the MCHC levels between the control group and all the experimental groups, that is Polyglactin 910 day 7, Polyglactin 910 day 28, DS clip day 7 and DS clip day 28.

A statistically significant difference was observed in the WBC measured between the control group and the DS clip group on day 7 and the DS clip group on day 28, as well as between the Polyglactin 910 group on day 7 and the DS clip group on day 7 and day 28. The WBC values measured in the Polyglactin 910 group on day 28 differed statistically significantly from the WBC values measured in the DS clip group on day 7, and the DS clip group on day 28.

There were statistically significant differences in the values of lymphocytes and neutrophils measured between the same groups, that is, between the control group and the Polyglactin 910 group on day 7 and the DS clip group on day 28, as well as between the Polyglactin 910 group on day 7 and the DS clip group on day 7.

Discussion

Rats are laboratory animals with low blood volume values in their cardiovascular system in proportion to their body weight [12]. A small loss of blood can lead to a lethal outcome in this animal. As a result, healthy rodents have higher PLT values than other larger species [13]. Their task is to react promptly to maintain normal haemostasis of the blood, which represents a form of physiological adaptation of the organism [14]. In our study, the PLT count in the DS clips group on days 7 and 28 was significantly lower than in the control group, as well as the Polyglactin 910 experimental group. The values obtained were even lower than the reference intervals [13].

Recent research into non-resorbable clips, titanium stapler clips and plastic hemo-o-lok clips regarding haematological parameters after laparoscopic appendectomy indicated the beginnings of hypochromic anaemia with the evident presence of anulocytes, or hypochromic erythrocytes [1,3]. Our study with the use of titanium DS clips in appendectomy also confirmed the beginnings of hypochromic anaemia. The most probable reason for this minimal loss of HGB, RBC and HCT was the course of the surgical procedure. There was clearly controlled bleeding, and the *regio abdominalis* in rats is extremely well vascularized [15]. Other erythrocyte indexes, such as MCV, MCH and MCHC, moved within a wide range, also with broad standard deviations, whereby the values established indicated hypochromic anaemia. The haematological values obtained from the DS stapler group indicated the very beginnings of anaemia, whilst the values in the Polyglactin 910 group indicated a somewhat more advanced stage of the same form of anaemia.

Recent reports have indicated that placing bioactive materials in tissue parts in an *in vivo* situation leads to a biological response [16]. In the practice of laparoscopic surgery, these are most frequently endoloop polyglactin 910 ligature sutures, as well as, for example, plastic and/or metal material [17]. In these circumstances, a certain reaction by the organism through leukocyte cells is expected. Recent research by Bajrić et al.[3] showed that non-resorbable titanium linear stapler clips cause a smaller reaction in the surrounding tissue than resorbable endoloop ligatures.

Placing DS titanium clips to close the appendiceal base in our study did not cause an increase in the total WBC count, in fact, the values obtained on days 7 and 28 were even lower than the control values. Although the values for WBC in the Polyglactin 910 group on days 7 and 28 were somewhat higher than the control values, they did not go beyond the upper physiological threshold for rats [18].

The leucogram results in our study correspond completely with the results of Bajrić et al.[3]. Neutrophilia was recorded in both experimental groups, where it was somewhat more marked in the Polyglactin 910 group.

This neutrophilia is an expected reaction in the peripheral blood in the post-operative period, due to the possible occurrence of an inflammatory process, the partial loss of blood, and the emotional stress in the operated experimental animals. All of this leads to the redistribution of neutrophils from the bone marrow reserves into the bloodstream [19].

Since rats have an extremely lymphocytic blood count [20], these values were in a slight decline in our study. The values obtained from both experimental groups were significantly lower than the control and were also below the lower physiological threshold [12].

The lymphopenia found in our study is completely in line with similar researches by Bajrić et al.[1,3]. The reasons for the occurrence of lymphopenia may be seen in the redistribution of lymphocytes as a response to the stressful situation for the experimental rates during and after the appendectomy [1,3]. The values obtained for monocytes, basophils, and acidophils were low and appropriate to their physiological variations, and there was no significant deviation.

It is important to point out the limitations of this study since it was performed on a rat model. The important question is how far these results may be applied to humans. It is also not possible to determine how far the short incision on the anterior abdominal wall and closing it provoked an increased loss of HGB and RBC. Another limitation of this study is the small sample, that is, the very likely possible repercussions on the results obtained due to the small sample.

However, we can conclude that the results of this study indicate that titanium DS clips, in comparison with the results obtained recently for linear titanium staplers, from the point of view of haematological indicators, are equally less unfavourable, in comparison with other methods such as plastic Hemo-o-lok clips and resorbable endoloop polyglactin 910 ligatures, used for laparoscopic closure of the appendiceal stump, or even some other surgical procedures on the abdominal tissue.

Financial Support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-forprofit sectors.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Experimental Design was conceived by A.B., M.K. and S.D. Data was collected by A.K., M.Č., M.K. and Aj.B. Statistical analysis was conducted by A.S. and A.K. Original draft was written by M.K. S.D, and N.H.A.

A.Z., D.R., M.S., M.Č., A.B., S.D., M.K. A.K.and Aj.B. are the executors of the experimental design and experimental research of this study. A.S. and N.H.A. finished data analysis and writing the first draft of the paper. M.Č. and M.S.participated in analysis of experimental results. S.D.and A.B. are the designers and leaders of the project, guiding experimental design, data analysis, thesis writing and revision. All authors have contributed to the revision and final proof-reading of the manuscript.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 617-624, 2021
DOI: 10.9775/kvfd.2021.25972

RESEARCH ARTICLE

Computed Tomographic Imaging Characteristics of the Thyroid Glands in Clinically Normal Van Cats

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Article ID: KVFD-2021-25972 Received: 03.05.2021 Accepted: 24.08.2021 Published Online: 27.08.2021

Abstract

This study was performed to obtain morphometric and volumetric measurements of thyroid glands of Van Cats, and to determine homotypical variations and biometric differences between the genders. Thyroid gland computed tomography (CT) images obtained from a total of 16 clinically healthy adult Van Cats (8 males, 8 females) were used in the study. Measurements were made on the transverse, sagittal, and dorsal sections of both right and left thyroid gland CT images. Volumes of the thyroid lobes were estimated using transverse ellipse, maximum ellipse, and planimetry methods. Statistical analyses were then performed on the obtained morphometric and volumetric values. In terms of sexual dimorphism in Van Cats, morphometric and volumetric measurement values of the thyroid gland were found to be larger in male cats than in females ($P<0.05$). When the parameters were inspected in terms of homotypical variations, measured values for the right thyroid lobes were found to be larger compared to the left thyroid lobe values in both male and female cats. Average length, width, height values for male thyroid glands were 25.41±1.63 mm, 2.49±0.09 mm, and 4.54±0.21 mm, respectively, while the same values were 19.57±0.92 mm, 2.06±0.17 mm, and 4.19±0.21 for female cats, respectively. Average thyroid gland volumes calculated through transverse ellipse, maximum ellipse, and planimetry values were 149.46±19.71 mm^3, 154.80±19.70 mm^3, and 166.86±19.04 mm^3 for males, respectively. The female counterparts for these values were 89.52±11.82 mm^3, 92.31±11.90 mm^3, 109.40±6.02 mm^3, respectively. As a result, various parameters on CT images of thyroid glands in clinically healthy Van Cats were measured and the results were statistically evaluated between the genders. It is thought that the results will be useful in the evaluation of abnormal thyroid glands in Van Cats CT images in clinical practices.

Keywords: *Computer tomography, Morphometry, Thyroid gland, Van Cat, Volumetric measurement*

Klinik Olarak Normal Van Kedilerinde Thyroid Bezinin Bilgisayarlı Tomografi Görüntülerinin Özellikleri

Öz

Bu çalışma, Van Kedilerinde thyroid bezinin morfometrik ve volümetrik ölçülerini elde etmek ve bu ölçüm değerlerinin hem homotipik varyasyonlar hem de cinsiyetler arasındaki biyometrik farklılıklarını ortaya koymak amacıyla yapıldı. Çalışmada klinik olarak sağlıklı 16 adet erişkin Van Kedisi (8 erkek, 8 dişi) thyroid bezine ait bilgisayarlı tomografi (BT) görüntüsü kullanıldı. Sağ ve sol thyroid bezine ait transversal, sagital ve dorsal kesitlerden morfometrik ölçümler alındı. Thyroid loblarının hacimleri transversal elips, maximum elips ve planimetri yöntemleri kullanılarak tahmin edildi. Morfometrik ve volümetrik değerlerin istatistiki analizi yapıldı. Van Kedilerinde seksüel dimorfizm bakımından, thyroid bezine ait morfometrik ve volümetrik ölçüm değerlerinin erkek kedilerde dişilere göre daha yüksek olduğu görüldü ($P<0.05$). Ayrıca, ölçüm parametrelerine homotipik varyasyonlar bakımından bakıldığında ise hem erkek hem de dişi kedilerde sağ loptaki ölçüm değerlerinin sol loba oranla daha yüksek olduğu tespit edildi. Thyroid bezine ait ortalama uzunluk, genişlik ve yükseklik değerleri erkeklerde sırasıyla 25.41±1.63 mm, 2.49±0.09 mm ve 4.54±0.21 mm, dişilerde ise bu değerler sırasıyla 19.57±0.92 mm, 2.06±0.17 mm ve 4.19±0.21 mm olarak belirlendi. Transversal elips, maximum elips ve planimetri yöntemleriyle hesaplanan thyroid bezine ait ortalama volüm değerleri erkeklerde sırasıyla 149.46±19.71 mm^3, 154.80±19.70 mm^3 ve 166.86±19.04 mm^3, dişilerde ise ortalama sırasıyla 89.52±11.82 mm^3, 92.31±11.90 mm^3 ve 109.40±6.02 mm^3 olarak hesaplandı. Sonuç olarak, klinik olarak sağlıklı Van Kedilerinde thyroid bezine ait ölçüm parametrelerinin istatistiksel olarak cinsiyetler arasındaki farklılıkları BT görüntüleri kullanılarak değerlendirildi. Elde edilen verilerin Van Kedilerinde anormal thyroid bezine ait BT görüntülerinin değerlendirilmesinde faydalı olacağı düşünülmektedir.

Anahtar sözcükler: *Bilgisayarlı tomografi, Morfometri, Thyroid bezi, Van Kedisi, Volümetrik ölçüm*

How to cite this article?

Yılmaz O: Computed tomographic imaging characteristics of the thyroid glands in clinically normal Van cats. *Kafkas Univ Vet Fak Derg,* 27 (5): 617-624, 2021.
DOI: 10.9775/kvfd.2021.25972

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Introduction

Living in the Van province region of Turkey and taking their names from it, Van Cats are an endemic species with a soft and furry body, occasional heterochromia (both eyes can be yellow or blue, or one eye can be blue while the other one is yellow), erect ears, round face, a triangular head shape. Van Cats enjoy playing in and around water, are capable hunters, and are quite intelligent. Due to these outstanding properties, Van Cats have long gained the attraction and affection of humans throughout history [1,2]. It has been reported that the average body length (centimeter: cm), tail length (cm), withers height (cm), breast girth (cm), and body weight (grams: g) measurement values of adult Van Cats are 38 cm, 27 cm, 31 cm, 30 cm, and 3992 g in males, and 36 cm, 26 cm, 27 cm, 31 cm, and 3132 g in females, respectively [3].

The thyroid glands are often bilaterally located on the ventral surface of the cranial section of the trachea, while occasionally they reach above the larynx [4]. The morphology of the thyroid gland varies between species. In cats and dogs, thyroid glands consist of two flat lobes right below the caudal of the larynx and on the lateral surface of the trachea, stretching alongside each other [4,5]. While cats usually lack an isthmus that connects these two lobes [6,7], occasionally a narrow and thin isthmus has been reported which connects the caudal ends of the lobes [4,8]. In dogs, usually in larger breeds, a thin isthmus can be found, albeit inconsistently and infrequently [9]. Hormones synthesized by this endocrine gland play role in sexual development, various metabolic activities, and other basic cellular activities [8].

Medical imaging processes like ultrasonography, thyroid scintigraphy, CT, and magnetic resonance imaging (MRI) are widely used in small pet animals like cats and dogs when attempting to inspect the thyroid gland anatomy and to identify, diagnose, stage, and manage thyroid gland diseases [7,9,10-12]. Additionally, CT and MRI methods, in particular, are being frequently employed to evaluate pathological conditions that influence the thyroid gland, like congenital, idiopathic, and spontaneous hypothyroidism and hyper-thyroidism cases, unilateral or bilateral diffuse or localized hyperplastic masses, or neoplasia, tumor, carcinoma, thyroid nodules and lesions that may occur due to a wide range of causes. These methods also allow for more detailed inspection and evaluation of the normal structure of the thyroid gland [7,11,13]. These imaging methods can also be used to obtain transverse, sagittal, and dorsal sections of the thyroid gland, which in turn help understand the anatomic structure of the gland more accurately [7,13].

Numerous studies have been performed to use the CT images to examine the anatomic structure of the cats' thyroid gland, determine its morphological and morphometric properties, and evaluate various pathologic alterations that may occur in the gland [6,7,13,14]. The present study was performed to obtain morphometric and volumetric measurements of thyroid glands of Van Cats, and to determine homotypical variations and biometric differences between the genders.

Material and Methods

Animals

A total of 16 adults and clinically healthy Van Cats (8 males and 8 females) obtained from the Van Yüzüncü Yıl University (YYU) Van Cat Research and Application Center in this study were used as the live material of the study. The cats were aged between 3 and 8 years old (the average ages of male and female cats were the same, 5±2 years), and their body weights (kilograms: kg) varied between 4.81 kg and 7.05 kg (the average body weights for male and female cats were 6.08±0.70 kg and 5.28±0.28 kg, respectively). Only cats without thyroid gland pathology history which were clinically healthy in terms of complete blood count, serum thyroid hormone levels, serum T4 concentration, serum biochemistry, and urine analysis results were included in the study. Thyroid glands on both sides of the cats were evaluated. The study was performed with the Van YYU Animal Experiments Local Ethics Committee (VAN YUHADYEK) decision (Approval no: 2020/09-07, date: 24.09.2020).

Anesthesia

Dissociative agents were used to place the cats under anesthesia. A combination of Ketamine (15 mg/kg, 10% injectable, IM) and Xylazine (1-2 mg/kg, 2% injectable, IM) was used to this end.

Computed Tomography Imaging

Van Cats were placed into the tray of a 16-detector multi-section CT device (Siemens, Somatom Sensation 16, Erlangen, Germany) in the symmetrical sternal recumbency position to obtain the CT images of their glandula thryoidea (gl. thryoidea). The imaging para-meters of the device were set as follows: resolution: 512×512 pixels, physical detector collimation: 16×0.6 mm, section thickness: 0.5 mm, kernel: U90u, gantry rotation period: 420 ms, rotation time(sec)/effective mAs/KV: 0.75/120/120, final section collimation: 32×0.63 mm; feed/rotation: 6 mm, and increment: 0.5 mm. The applied CT doses and scans were performed considering the standard protocols, based on the literature [15,16]. Obtained CT images were saved in DICOM (Digital Imaging and Communication in Medicine) format, which were then transferred to CD.

Obtaining Thyroid Gland Measurements From Computed Tomography Images

Measurements on images were performed in the computer environment using RadiAnt DICOM Viewer (64 Bit) software. Electronic calipers were used on the transverse, sagittal, and dorsal section images of the Van Cats' right and left

thyroid glands to obtain the measurements. During the measurements, the maximum length and height of each thyroid lobe in each section view (transverse, sagittal, and dorsal) were considered valid. Transverse images were used to determine the thyroid lobe length, where the number of image slices containing thyroid tissue was multiplied by the slice thickness (0.5 mm). Measurements were recorded in millimeter (mm) units.

The volumes of the thyroid lobes were calculated using three different methods. The first method was the planimetry method, which is a stereological approach where the edges of the gland were traced out by hand over the transverse section image in each slice to determine the corresponding area. The total volume of the thyroid gland was then calculated multiplying the area with the slice thickness, and adding all of the results together. The second method was the transverse ellipse volume method, where the transverse section images alone were used to obtain length, width, and height values placed into the ellipse volume formula (Volume = length x width x height x 0.524). The third method was the maximum ellipse volume method, which is similar to the second method but considers the linear measurement values for length, width, and height parameters in each slice. The total thyroid gland volume for each cat was obtained by adding the right and left thyroid lobe volumes together. To evaluate the repeatability of the values, each of the measurements was made three times by the same anatomist and the average of these values was used.

Statistical Analysis

In the present study, each of the sexes was represented by 8 cats, and the sampling size power (test strength) was calculated as 90%. Definitive statistics were continuous variables in the study were expressed as mean, standard deviation, minimum, and maximum values. Shapiro-Wilk (n<50) and Skewness-Kurtosis tests were used to control if the continuous measurement averages displayed a normal distribution, and since the variables were found to have nominal distribution, the parametric tests were used. Comparison of the measurements based on the genders was performed using the independent t-test. Comparison of right and left thyroid glands (separately for each gender) was performed using paired t-test. Pearson correlation coefficients were calculated to determine potential correlations between the measurements (separately for each gender). Statistical significance level was considered as 5% for the calculations, which were performed using SPSS (IBM SPSS for Windows, ver.24) statistical package software.

Results

In the present study, morphometric measurements of the Van Cat thyroid glands were taken from the transverse (length, height, width), dorsal (length, width) and sagittal (length, height) sections of CT images *(Fig. 1, Fig. 2, Fig. 3)*. Volumetric values for the thyroid glands were also obtained using three different methods of planimetry, transverse, and maximum ellipse volume methods. The measurements obtained were evaluated in terms of sexual dimorphism and homotypical variations, and the results are provided in *Table 1, Table 2,* and *Table 3*. Any statistically significant correlations between the measurements were noted (P<0.05).

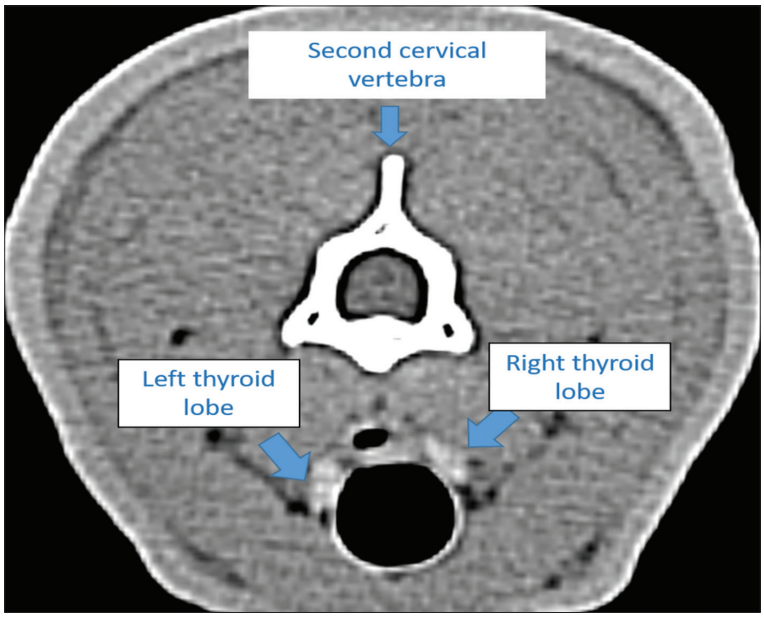

Fig 1. Transverse CT image of the right and left thyroid glands at the middle aspect of the second cervical vertebra in a clinically normal Van Cat. CT images were viewed at a window width (WW) of 300 Hounsfield Unit (HU) and a window level (WL) of 50 HU

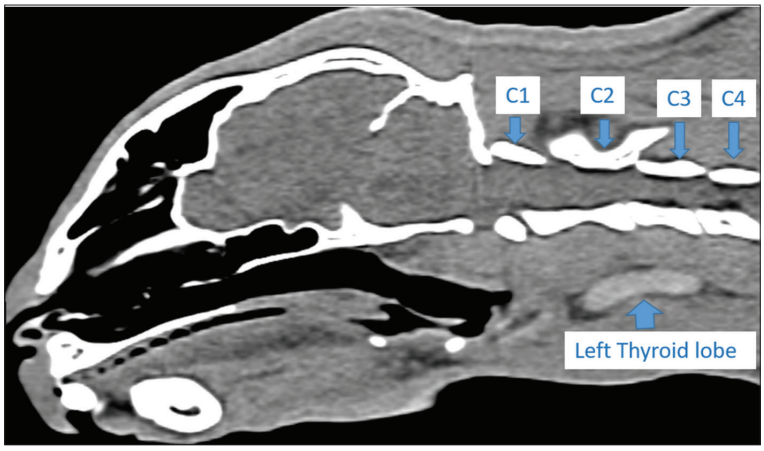

Fig 2. Sagittal CT image of the left thyroid gland in a clinically normal Van Cat. CT images were viewed at a window width (WW) of 400 Hounsfield Unit (HU) and a window level (WL) of 50 HU

Table 1 represents to definitive statistics for the transverse, sagittal, and dorsal section morphometric measurement values between the right and left lobes, and per gender. Accordingly, right and left transverse length, right and left transverse height, right and left width, right and left sagittal length, right and left sagittal height, right and left dorsal length, and right and left dorsal width measurements were found to be higher in male cats, compared to females (P<0.05). Furthermore, evaluation of the definitive statistics for the right and left thyroid lobes for the genders have shown that both male and female cats had higher values for their right thyroid lobe, compared to their left counterparts. On that note, female cats were found to have statistically higher right-side lobe transverse length and width, sagittal length, and dorsal length and width values compared to their left-side lobe, while male cats were found to have statistically higher right-side lobe transverse length and height, sagittal length and height, and dorsal length and width values compared to their left-side lobe (P<0.05). Average length, width, height values for all the sections (taking into consideration both right and left side thyroid lobes) were found as 25.41±1.63 mm, 2.49±0.09 mm, and 4.54±0.21 mm for males, and as 19.57±0.92 mm, 2.06±0.17 mm, and 4.19±0.21 mm for females, respectively.

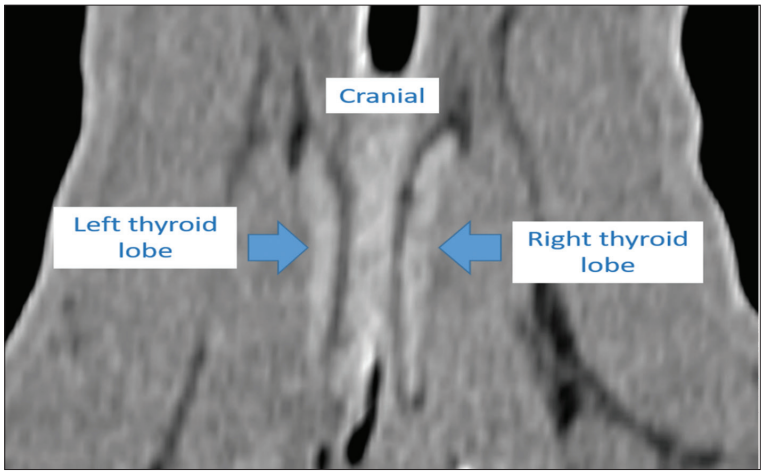

Fig 3. Dorsal CT image of the right and left thyroid glands in a clinically normal Van Cat. CT images were viewed at a window width (WW) of 400 Hounsfield Unit (HU) and a window level (WL) of 50 HU

Definitive statistics and right and left side comparisons for the Van Cat thyroid gland volumetric measurements based on gender are provided in *Table 2*. When the thyroid gland volumes obtained using transverse ellipse, maximum ellipse, and planimetry methods are evaluated, it was noticed that all three methods agreed on the result that male cats had statistically larger glands compared to females (P<0.05).

Table 1. *Descriptive statistics and homotypic variations of the morphometric measurement values obtained using CT images of the thyroid gland in Van Cats by gender*

Imaging Plane			Female			Male			P
			Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	
Transverse	Length	Right	20.25±0.91#	18.40	21.20	25.80±1.71#	24.00	28.80	0.001*
		Left	19.40±1.00	17.60	20.80	25.35±1.60	23.60	27.60	0.002*
	Width	Right	2.07±0.14#	1.91	2.33	2.47±0.07	2.41	2.61	0.001*
		Left	1.99±0.19	1.68	2.28	2.45±0.08	2.37	2.60	0.002*
	Height	Right	4.26±0.14	4.09	4.43	4.56±0.20#	4.36	4.94	0.004*
		Left	4.19±0.20	3.88	4.46	4.49±0.22	4.25	4.91	0.012*
Sagittal	Length	Right	19.90±0.82#	18.30	20.70	25.55±1.69#	23.60	28.50	0.001*
		Left	19.01±0.93	17.20	20.30	25.13±1.55	23.20	27.30	0.002*
	Width	Right	NA						
		Left							
	Height	Right	4.18±0.23	3.97	4.57	4.60±0.21#	4.39	4.97	0.002*
		Left	4.11±0.25	3.80	4.42	4.51±0.21	4.28	4.93	0.004*
Dorsal	Length	Right	19.88±0.88#	18.20	20.70	25.49±1.68#	23.60	28.40	0.001*
		Left	18.98±0.96	17.10	20.30	25.15±1.54	23.40	27.50	0.002*
	Width	Right	2.13±0.15#	2.00	2.41	2.54±0.09#	2.44	2.68	0.001*
		Left	2.04±0.20	1.77	2.40	2.49±0.11	2.35	2.65	0.002*
	Height	Right	NA						
		Left							

** Shows the difference between the female-male (*P<0.05: Independent T-test); # Shows the difference between right and left separately in genders (#P<0.05: Paired T-test); NA = Not applicable*

Table 2. *Descriptive statistics and homotypic variations of the volumetric measurement values obtained using CT images of the thyroid gland in Van Cats by gender*

Method		Female			Male			P
		Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	
Transverse ellipse	Right	93.92±9.77#	78.45	106.46	152.51±20.18#	135.13	186.47	0.001*
	Left	85.11±14.11	60.12	104.44	146.40±19.48	127.78	184.63	0.002*
	Both	89.52±11.82	69.28	105.45	149.46±19.71	131.46	185.55	
	Total	179.04±23.64	138.57	210.90	298.91±39.42	262.91	371.10	
Maximum ellipse	Right	97.03±9.74#	82.15	110.12	158.86±19.88#	138.03	192.63	0.001*
	Left	87.58±14.35	63.34	109.93	150.74±19.68	129.50	188.94	0.002*
	Both	92.31±11.90	72.74	110.03	154.80±19.70	133.77	190.79	
	Total	184.61±23.79	145.48	220.05	309.61±39.39	267.54	381.58	
Planimetry	Right	112.05±5.09#	106.25	122.63	170.24±19.03#	152.64	199.80	0.001*
	Left	106.75±7.19	100.94	120.51	163.48±19.16	144.74	195.21	0.002*
	Both	109.40±6.02	103.85	121.57	166.86±19.04	149.76	196.70	
	Total	218.80±12.04	207.70	243.14	333.72±38.09	299.53	393.40	

** Shows the difference between the female-male (*P<0.05: Independent T-test); # Shows the difference between right and left separately in genders (#P<0.05: Paired T-test); Both=Mean volume of left and right thyroid lobes. Total=Mean volume of thyroid tissue in each cat*

Table 3. *The correlation between morphometric and volumetric measurement values of the thyroid gland with age and body weight in Van Cats by gender*

Measurement Parameters			Female		Male	
			Age (5±2 years)	Body Weight (5.28±0.28 kg)	Age (5±2 years)	Body Weight (6.08±0.70 kg)
Transverse length	Right	r	0.473	0.106	0.919**	0.908**
	Left	r	0.598	0.455	0.929**	0.920**
Transverse width	Right	r	0.877**	0.440	0.871**	0.825*
	Left	r	0.836**	0.179	0.710*	0.682*
Transverse height	Right	r	0.347	0.185	0.894**	0.756*
	Left	r	0.752*	0.413	0.864**	0.720*
Sagittal length	Right	r	0.234	0.013	0.909**	0.912**
	Left	r	0.443	0.388	0.906**	0.908**
Sagittal height	Right	r	0.311	0.120	0.908**	0.924**
	Left	r	0.669	0.249	0.875**	0.881**
Dorsal length	Right	r	0.106	-0.171	0.908**	0.910**
	Left	r	0.355	0.333	0.921**	0.912**
Dorsal width	Right	r	0.741*	0.362	0.522	0.503
	Left	r	0.847**	0.246	0.590	0.586
Transverse ellipse volume	Right	r	0.902**	0.401	0.970**	0.906**
	Left	r	0.892**	0.378	0.952**	0.885**
Maximum ellipse volume	Right	r	0.844**	0.377	0.960**	0.929**
	Left	r	0.906**	0.423	0.938**	0.901**
Planimetry volume	Right	r	0.883**	0.539	0.967**	0.934**
	Left	r	0.895**	0.570	0.967**	0.945**

*** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level, r: Pearson correlation coefficients*

Average volume values that calculated using transverse ellipse, maximum ellipse, and planimetry methods for male cats were found as 149.46±19.71 mm^3; 154.80±19.70 mm^3; 166.86±19.04 mm^3, while the same values were found as 89.52±11.82 mm^3, 92.31±11.90 mm^3, 109.40±6.02 mm^3 for female cats, respectively.

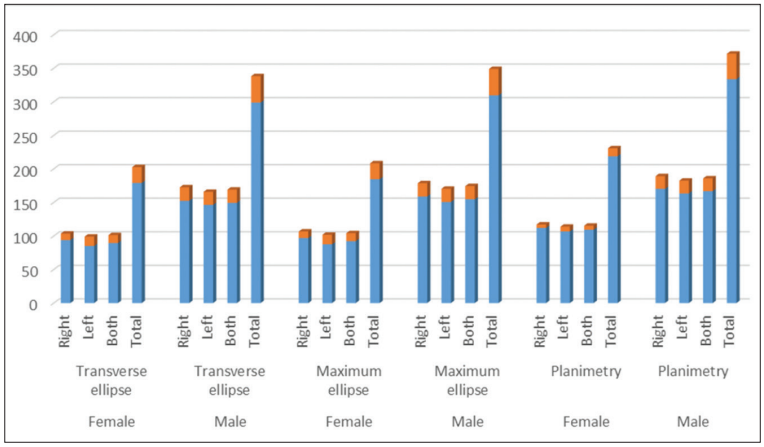

Fig 4. Distribution of the thyroid glands volumetric measurements in Van Cats by gender

Distribution of the thyroid glands volumetric measurements by gender has been given in *Fig. 4*. Generally, according to the graph in the figure, it was seen that male Van Cats were higher volumetric measurement of thyroid gland compared to female Van Cats.

Table 3 displays the correlation between the morphometric and volumetric measurement values for thyroid glands for each gender with other parameters like age and body weight. Accordingly, a positive correlation was observed between the female cats' age with right - left transverse width, left transverse height, right - left dorsal width, right - left transverse ellipse volume, right - left maximum ellipse volume, and right - left planimetry volume values ($P<0.05$). A further and generally positive correlation was determined between female cats' body weights with thyroid gland morphometric and volumetric values (except for right - side dorsal length), but the correlation was not statistically significant ($P>0.05$). In male cats, on the other hand, both age and body weight were found to have a positive and statistically significant correlation with right - left transverse length, right - left transverse width, right - left transverse height, right - left sagittal length, right - left sagittal height, right - left dorsal length, right - left transverse ellipse volume, right - left maximum ellipse volume, and right - left planimetry volume values ($P<0.05$).

Discussion

While methods like high-resolution ultrasonography and scintigraphy imaging are effective in determining primary thyroid and parathyroid disorders, CT and MRI methods are becoming more widely and effectively used in determining a variety of cases that influence the thyroid gland. These situations particularly include determination of the size and operative conditions of masses in thyroid glands like ectopic thyroid, thyroidal masses, or aggressive thyroid neoplasia, and MRI and CT imaging are also capable of providing aid detailed inspection of the normal structure of the thyroid gland [11,13]. Furthermore, morphometric and volumetric measurement values for small pet animals like cats and dogs obtained using imaging methods like CT and MRI scans provide valuable information for Veterinary clinicians in the diagnosis and treatment of various pathological conditions concerning the thyroid gland and neighboring anatomical structures [7,11,13,14,17]. The present study is a study that determines the morphometric and volumetric values for right and left-side of thyroid lobes in Van Cats using computed tomography, and that reveals the biometric differences between male and female cats in these values.

In general, measurement values for the thyroid gland obtained using medical imaging methods have been studied in humans, dogs, and cats in terms of homotypical variations and sexual dimorphism [7,9,18-20]. In terms of sexual dimorphism, it has been determined with the present study that morphometric and volumetric measurement values for male cats are larger compared to female cats. Evaluation of the homotypical variations in the parameters, on the other hand, has shown that right-side of thyroid gland measurement values are higher compared to the left-side of counterpart. These findings lead us to conclude that in Van Cats, the right lobe of the thyroid gland is larger compared to the left lobe, and male cats have larger thyroid glands compared to females.

Dyce et al.[5] have reported that the thyroid lobes in medium-sized dogs are approximately 5 cm in length and 1.5 cm in width, and that immature and brachiocephalic species had larger glands, while in cats each thyroid gland lobe is approximately 2 cm in length and 0.3 cm in width. Drost et al.[7] have used helical computed tomography to perform a clinical study on 8 female cats to obtain transverse, sagittal and dorsal sections on both right and left thyroid lobes, and reported the average length, height, and width values as 16.78±2.48 mm, 4.19±0.93 mm, and 2.28±0.76 mm, respectively. In the present study, the average length, width, and height values in all sections for right and left thyroid glands were found as 25.41±1.63 mm, 2.49±0.09 mm, and 4.54±0.21 mm for male cats respectively, and as 19.57±0.92 mm, 2.06±0.17 mm, and 4.19±0.21 mm for female cats, respectively. These values and the literature in general, and any small variations are attributed to age, length, body weight, and racial properties of the studied cats. Meanwhile, various studies have reported that these sizes change significantly in cats with hyperthyroidism. For instance, Volckaert et al.[21] performed a study where they reported the length, height, and width of the thyroid glands of cats with hyperthyroidism as 26.7 mm, 10.5 mm, and 13.3 mm respectively with ultrasonography, and as 20.7 mm, 13.2 mm, and 13.1 mm respectively with

scintigraphy. Besides, Wisner et al.[22] reported right and left side length, height, and width values in hyperthyroid cats as 21.9±4.4 mm, 20.2±3.6 mm; 8.1±3.0 mm, 5.5±2.4 mm; 7.7±2.4 mm, 5.7±2.1 mm, respectively. These literature results can be interpreted as cats having a large variation in scale in terms of hyperthyroidism.

Certain studies performed on humans have reported a generally positive correlation between the size of the thyroid gland with age and body weight [19,23]. On the other hand, Lee et al.[18] have reported that humans have a strong negative correlation between thyroid measurements and age, but have a positive correlation between body weight and thyroid gland size. Furthermore, Taeymans et al.[9] used CT images to determine the normal canine thyroid gland sizes and reported a negative yet insignificant correlation between age and gland size. The same researchers also reported that no meaningful correlations existed between body weight and thyroid gland size. Despite that, Taeymans et al.[10] performed another study using MRI and reported a correlation between normal canine thyroid gland size and body weight. In the present study, however, a generally positive correlation was determined between male cats' thyroid measurements and their age and body weights. While female cats were found to have a mostly positive correlation between age and thyroid measurements, no correlation was determined between their body weight and thyroid sizes. Based on these results it can be concluded that correlations between age and body weight and thyroid gland measurements can vary between species, and even within the same species variations may emerge due to factors like length, racial properties, measurement methods, and iodine intake.

Many studies were performed on cats and dogs to determine volumetric measurements of thyroid glands using medical imaging methods like ultrasonography, scintigraphy, and computed tomography [7,9,21,22]. The average thyroid gland volume of clinically healthy dogs was determined as 1148.04 mm^3 in the precontrast period, while it was determined as 1188.88 mm^3 in the postcontrast stage [9]. In healthy cats, ultrasonography was used to determine the lobar volume value of the thyroid gland in cats as 85 mm^3, and total volume as 169 mm^3 [22]. In another study, computed tomography images were used in conjunction with a transverse ellipse, maximum ellipse, and sum of areas methods to obtain average thyroid gland volume, which was reported as 75.06±31.52 mm^3, 115.63±50.80 mm^3, and 113.75±49.46 mm^3 for the methods, respectively [7]. In the present study, transverse ellipse, maximum ellipse, and planimetry methods were used to calculate the thyroid gland average volumes, which were found as 149.46±19.71 mm^3, 154.80±19.70 mm^3, and 166.86±19.04 mm^3 for the methods respectively in male cats, and as 89.52±11.82 mm^3, 92.31±11.90 mm^3, and 109.40±6.02 mm^3 in female cats, respectively. This variation between the measurements was attributed primarily to gender, followed by age, length, body weight, racial properties, and the differences between measurement methods.

There were a few limitations to this study. (1) The morphometric and volumetric measurement values of the thyroid gland of Van Cats in the study were obtained under general anesthesia and using a CT device. CT application has some disadvantages such as the harmful effects of ionizing radiation applied on the body in CT scans. However, medical imaging methods such as 2D and 3D ultrasound, which provide the opportunity to obtain images for morphometric and volumetric measurements of the thyroid gland of Van Cats, without putting the cats under general anesthesia and exposing them to ionizing radiation, were not used in the study. (2) It would be optimal to compare CT measurements of the thyroid gland of Van Cats with in situ measurements obtained using calipers. However, all of the Van Cats used in the presented study were healthy and alive. CT images of the study were obtained only under general anesthesia without any damage to the vitality of these cats, and measurements were taken from these images using electronic calipers through the RadiAnt DICOM Viewer (64 Bit) software program. For these reasons, it was not possible to dissect the thyroid gland of Van Cats, which is an endemic species, to obtain measurement values by using a caliper or to examine histologically.

As a result, the morphometric and volumetric parameters of thyroid glands of clinically healthy Van Cats were measured using computed tomography images, and the sexual dimorphism and homotypical variations between the measurements were statistically determined. We humbly believe the results will be useful in the evaluation of thyroid gland pathologies in Van Cats for Veterinary clinicians.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 625-631, 2021
DOI: 10.9775/kvfd.2021.25998

RESEARCH ARTICLE

Shelf-life Estimation of Mullet *(Mugil cephalus)* Fillets by Mathematical Models Based on Some Biochemical Parameters and Sensory Evaluation

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Article ID: KVFD-2021-25998 Received: 12.05.2021 Accepted: 10.09.2021 Published Online: 25.09.2021

Abstract

This study aimed to estimate the shelf-life of mullet fillets stored in ice. Cadaverine, putrescine, total volatile base nitrogen (TVB-N), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were evaluated together with sensory analyses. Empirical mathematical models were used to describe the data, and shelf-life was estimated by using acceptability limit and the model proposed for each parameter. The models proposed were all in agreement with the experimental data and can be safely used to estimate the shelf-life of mullet fillets. The lowest shelf-life was found as 5.2 days for the sensory analysis performed by trained panelist. Shelf-lives were determined as 6.5 and 7.5 days according to TBARS and PV, respectively. Lipid oxidation seemed to be the reason of the results since mullet is considered as a medium-fat fish. Highest shelf-life was calculated as 11.7 days according to cadaverine which is an indicator of late spoilage due to late formation. The results presented here revealed that predictive modeling can be used to describe the kinetic data for fish quality and further to estimate the shelf-life.

Keywords: *Predictive modeling, fish, shelf-life, TVB-N, TBARS, Peroxide value*

Kefal *(Mugil cephalus)* Filetolarının Bazı Biyokimyasal Parametrelere ve Duyusal Değerlendirmeye Dayalı Matematik Modellerle Raf Ömrü Tahmini

Öz

Bu çalışmada buzda depolanan kefal filetolarının raf ömrü tahmini amaçlanmıştır. Kadaverin, putresin, toplam uçucu bazik azot (TVB-N), peroksit değeri ve tiyobarbitürik asit (TBARS) değeri duyusal analiz sonuçları ile birlikte değerlendirilmiştir. Bu verileri tanımlamak için deneysel matematiksel modeller kullanılmış, her parametre için kabul edilebilirlik sınırı ve önerilen model kullanılarak raf ömrü tahmin edilmiştir. Önerilen modellerin tümü deneysel verilerle uyum gösterdiğinden, kefal filetolarının raf ömrünü değerlendirmek için güvenle kullanılabilmektedir. En düşük raf ömrü duyusal analiz için 5.2 gün olarak bulunmuştur, ancak eğitimli panelistler tarafından gerçekleştirilen duyusal analizler gerçek tüketici davranışını temsil etmeyebilir. Raf ömrü TBARS'a göre 6.5 gün, PV'ye göre ise 7.5 gün olarak hesaplanmıştır. Kefal orta yağlı balık olarak kabul edildiğinden, bu sonuçların nedeninin lipid oksidasyonu olduğu düşünülmektedir. En yüksek raf ömrü ise geç bozulmanın indikatörü olarak kullanılan kadaverine göre 11.7 gün olarak hesaplanmıştır. Burada sunulan sonuçlar, tahminsel modellemenin balık kalitesi için kinetik verileri tanımlamak ve ayrıca raf ömrünü tahmin etmek için kullanılabileceğini ortaya koymaktadır.

Anahtar sözcükler: *Tahminsel modelleme, balık, raf ömrü, TVB-N, TBARS, Peroksit değeri*

INTRODUCTION

Fish and other seafood products are considered one of the most important food commodities due to their delicacy with high nutritive value, hence their consumption has risen substantially over the past few decades. Therefore, it is very important to ensure the safety of edible fish for global fishing industry [1,2]. Fish production is estimated to reach about 179 million tons in 2018 globally and 156 million tons (about 88%) ended up in our plates whereas

How to cite this article?

Pilavtepe-Çelik M, Buzrul S: Shelf-life estimation of mullet *(Mugil cephalus)* fillets by mathematical models based on some biochemical parameters and sensory evaluation. *Kafkas Univ Vet Fak Derg,* 27 (5): 625-631, 2021.
DOI: 10.9775/kvfd.2021.25998

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12% was used for non-food purposes. Live, fresh or chilled fish still represent the largest share of fish utilized for direct human consumption [3].

Striped mullet *(Mugil cephalus)* is an important and one of the most widely distributed seafood fish species around the world, occurring in the coastal waters of the tropical, subtropical and temperate zones of all seas. The fish has a good market in some countries, especially in the southern and eastern Mediterranean region. It is also consumed in many Asian countries. It is usually consumed fresh [4,5]. Fish and other seafood products are the most valuable nutrients, however, high levels of moisture, free amino acids, and unsaturated fatty acids make them extremely perishable foods [6]. These products are especially susceptible to chemical, enzymatic and microbiological spoilage during processing or storage [2]. Fresh and storage in ice are the common commercial practice for most fishes. Keeping fish in ice is one of the most efficient treatments for retarding spoilage [7].

The rate of spoilage during ice storage of fish depends on species, storage conditions, handling and processing from the catch to the consumer. Under improper conditions, fish are susceptible to changes in physicochemical properties and microbial spoilage. Therefore, maintaining and monitoring safety and freshness of fish are very important for consumers and food industry. There are many conventional techniques to point the state of freshness including sensory analysis, physical detection techniques, (bio)chemical and microbiological parameters such as total volatile basic nitrogen (TVB-N), thiobarbituric acid value (TBA), peroxide value (PV), biogenic amines and total viable counts (TVC). Most of these traditional techniques are time-consuming, expensive and tedious, requiring well trained operators [8-10].

Shelf-life determination of fresh fish is very important when assessing the quality. Unfortunately, because of many factors affecting freshness, the precise estimation of fish shelf-life is difficult and complex [11]. In shelf-life studies, (bio)chemical parameters and volatile compounds such as TVB-N can be monitored and modelled. In recent times, predictive modeling is a key component in quantitative risk assessment and management of seafood safety and quality.

Predictive models that are able to estimate quality aspects of fish and seafood products can be used as tools for quality management in the seafood industry [7]. Since a single parameter alone does not reflect the freshness during storage of fish, models resulting from the combination of several parameters (physical, microbiological, chemical, sensory attributes) could be more potent and comprehensive. Therefore, the main purpose of this study was to develop models in order to predict the shelf-life of striped mullet fillets stored in ice by using TVB-N, TBA, PV, biogenic amines (cadaverine and putrescine) and sensory analysis data.

Material and Methods

Data Set

All data used in this study were taken from a recent work published by Pilavtepe-Celik et al.[12] Experimental procedures (sample preparation, storage conditions, sensory evaluation, chemical analyses) were explained in detail by the authors. Briefly, biogenic amines (cadaverine and putrescine), total volatile base nitrogen (TVB-N), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were measured during storage (up to 13 days) at 0°C. Sensory analyses were performed with 11 trained panelists during storage (up to 11 days) at 0°C.

Modeling

To describe cadaverine and putrescine over time, following model was proposed:

$$C(t) = \ln\{1 + \exp[k \cdot (t - t_c)]\} \quad (1)$$

where $C(t)$ is the concentration of cadavarine or putrescine at a time t (day), t_c is the time level at which cadavarine/putrescine concentration starts to accelerate and k is the rate (time^{-1}) at which cadavarine/putrescine concentration climbs as the time passes to a level well above t_c. Note that according to this model initially i.e., t = 0 cadavarine or putrescine concentration is zero. This model was originally proposed by Campanella and Peleg [13] as the secondary model in microbial inactivation.

The same model with an intercept (C_0) incorporated was used to describe total volatile base nitrogen (TVB-N):

$$C(t) = C_0 + \ln\{1 + \exp[k \cdot (t - t_c)]\} \quad (2)$$

where $C(t)$ is the value of TVB-N at a time t (day), C_0 is the initial ($t = 0$) TVB-N value, t_c is the time level at which TVB-N value starts to accelerate and k is the rate (time^{-1}) at which TVB-N value climbs as the time passes to a level well above t_c.

For peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) the following model was used:

$$C(t) = C_{max} \cdot \left(1 + \frac{t_{max} - t}{t_{max} - t_{rate}}\right) \cdot \left(\frac{t}{t_{max}}\right)^{\frac{t_{max}}{t_{max} - t_{rate}}} \quad (3)$$

where $C(t)$ is the value of PV or TBARS at a time t (day), C_{max} is the maximum PV/TBARS value attained at time t_{max} and t_{rate} is the time at which maximum rate is achieved.

Finally, sensory evaluation was described by using simple linear model viz.,

$$C(t) = k \cdot t \quad (4)$$

where $C(t)$ is the sensory score at a time t (day), k is the

slope (time^{-1}) of the line which shows the rate of increase of the score.

Goodness-of-Fit Evaluation

Models were assessed by coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}) and root mean square error (RMSE) values. Higher R^2 and R^2_{adj}, and lower RMSE (compared to the *y* axis) values indicated good fit. SigmaPlot (Version 12.0, Chicago, IL) was used for linear and non-linear regression, and also to obtain the parameter values and goodness-of-fit indices.

Determination of Shelf-life

Putrescine and cadaverine are the biogenic amines commonly analyzed in seafood and their sum should be less than 20 mg/kg, or putrescine concentration should be less than 10 mg/kg [14,15]. Therefore, 10 mg/kg was used as the limit of consumption for cadaverine and putrescine to estimate shelf-life. One of the most common indicators of fish spoilage is TVB-N. Fish, according to TVB-N value, can be considered as spoiled above 35 mg/100 g fish [16,17] and this value was used as the limit value to calculate shelf-life. Upper limits of acceptability for PV and TBARS values were set to 1 mmole CPO/kg fish and 6.5 mg MDA_{eq}/kg fish, respectively for shelf-life prediction. Finally, for sensory evaluation rejection point was set to 5 out of 15 for overall quality parameter to estimate shelf-life of mullet fillets [12].

The shelf-life of fish samples were obtained as the intersection point of the model fits and the acceptability limits given above for each analysis. Therefore, shelf-life could be calculated by using the numerical values of the model parameters in the model equation and acceptability limit values. Moreover, intersections of the 95% confidence bands (upper and lower) and the acceptability limits were used as the 95% confidence interval of the shelf-life.

Results

Model Fits

Figure 1 shows the fit of Eq.(1) to cadaverine and putrescine, and *Fig. 2* shows the fit of Eq.(2) to TVB-N. The fit of Eq.(3) to PV and TBARS is shown in *Fig. 3* while the fit of Eq.(4) for sensory data is shown in *Fig. 4*. Confidence bands (95%) of each fit (black dashed lines) is also shown together with the maximum allowance of each analysis (gray dashed line) during the storage. *Table 1* and *Table 2* list the model parameters and goodness-of-fit indices. Fitted curves went near the data *(Fig. 1, 2, 3* and *4)*, and higher (and closer to 1.0) R^2, R^2_{adj} (≥0.9190) and lower RMSE (≤1.0064) values were obtained *(Table 1* and *Table 2)* indicating that models proposed were in good agreement with the experimental data. Moreover, parameter uncertainties (standard errors) were very low compared to parameter values so that all parameters were statistically significant ($P<0.05$).

Shelf-life Estimation

By using the parameter values of the models given in *Table 1* and *Table 2* and the maximum allowance level of each analysis, shelf-life of the mullet fillets was calculated. Furthermore, by using the 95% confidence bands of each fit 95% confidence intervals of shelf-life were also estimated. These are given in *Table 3*. The lowest shelf-

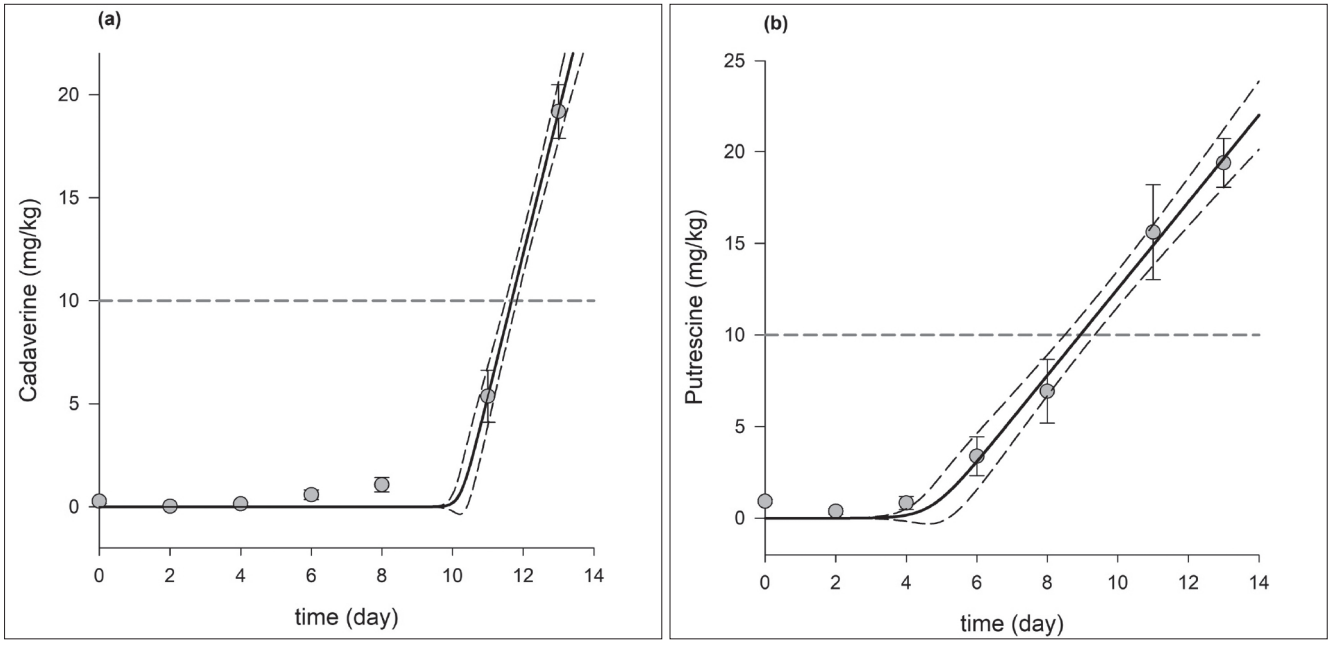

Fig 1. Change of cadaverine (a) and putrescine (b) in mullet fillets at 0°C with respect to time. Gray circles are the experimental data points; error bars represent standard deviation of three independent replications. Solid black line is the model fit [Eq. (1)], dashed black lines are the 95% confidence bands and dashed gray line is the limit for the shelf-life. Original data are from Pilavtepe-Celik et al.[12]

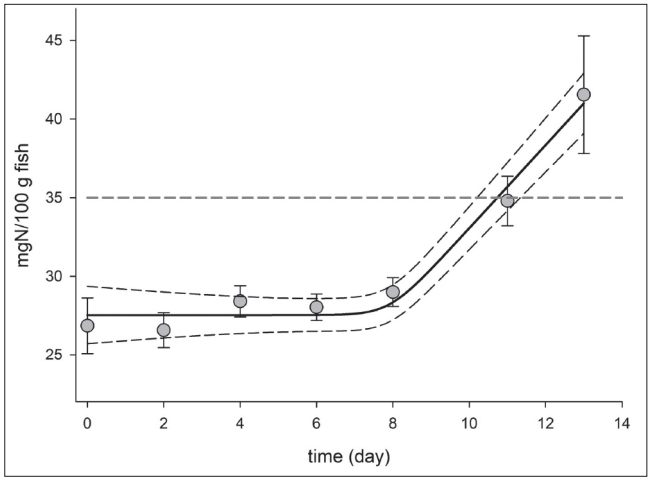

Fig 2. Change of TVB-N in mullet fillets at 0°C with respect to time. Gray circles are the experimental data points; error bars represent standard deviation of three independent replications. Solid black line is the model fit [Eq.(2)], dashed black lines are the 95% confidence bands and dashed gray line is the limit for the shelf-life. Original data are from Pilavtepe-Celik et al.[12]

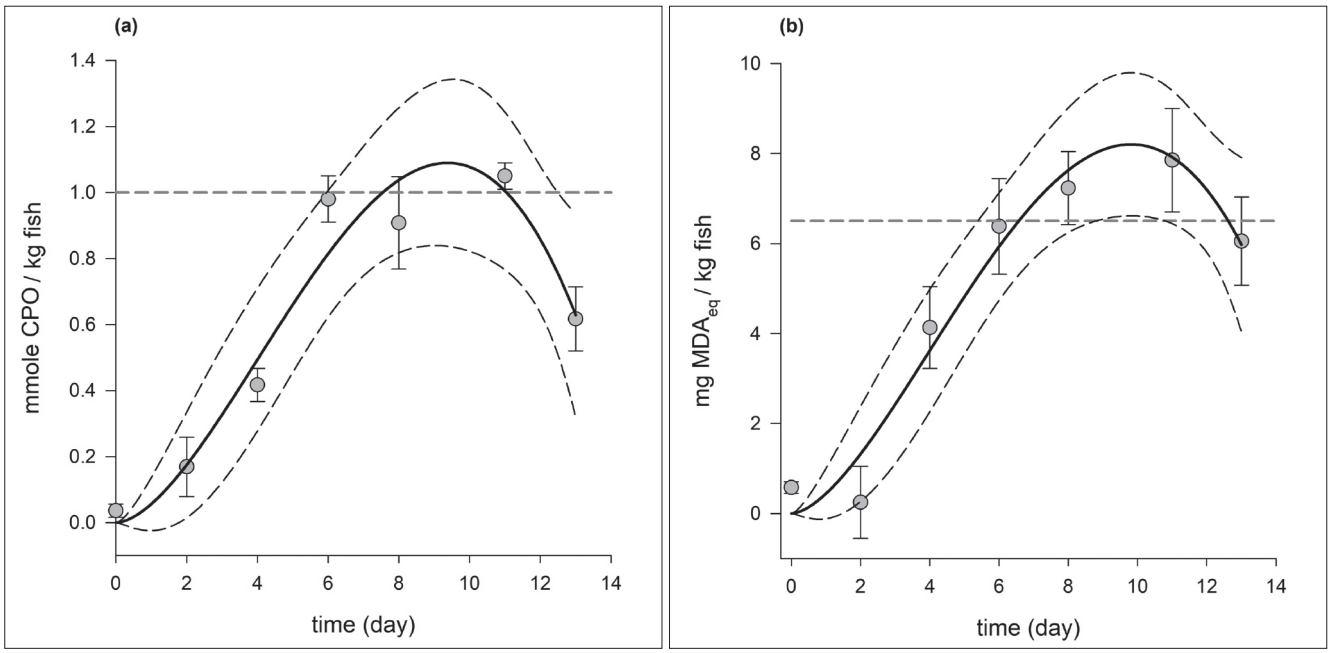

Fig 3. Change of PV (a) and TBARS (b) in mullet fillets at 0°C with respect to time. Gray circles are the experimental data points; error bars represent standard deviation of three independent replications. Solid black line is the model fit [Eq.(3)], dashed black lines are the 95% confidence bands and dashed gray line is the limit for the shelf-life. Original data are from Pilavtepe-Celik et al.[12]

life was determined in sensory analyses as 5.2 days with 95% confidence interval of 4.4 to 6.4 days followed by TBARS as 6.5 days with 95% confidence interval of 5.4 to 8.8 days. Highest one was obtained in cadaverine as 11.7 days (11.5 to 11.8 days) followed by TVB-N as 10.7 days (10.2 to 11.3 days) *(Table 3)*. Note that upper limit of PV cannot be calculated because the acceptability limit never intersected with the lower 95% band *(Fig. 3-a)*.

Discussion

Shelf-life of fish during storage is mainly dependent on storage temperature. Since the storage temperature (0°C) was lower than the refrigeration temperature (4°C) in this study, it may be expected to have longer shelf-life. However, the minimum shelf-life was estimated as low as 5.2 days: the lowest shelf-life was determined from the modeling of sensory analysis (5.2 days with 95% confidence intervals of 4.4-6.4 days) and this was followed by TBARS (6.5 days) and PV (7.5 days) *(Table 3)*. Some studies showed that the filleted fish has shorter shelf-life than whole fish since the filleting procedure causes cross-contamination and exposure of fish lipids to atmospheric oxygen leading to enhanced enzymatic activity and accelerated oxidation [18,19]. Average fat content of mullet fillets was found as about 5%, indicating medium-fat fish. Hence, quick deterioration or lipid oxidation was the main reason of low shelf-life even at 0°C. Both PV and TBARS increased and then decreased during the shelf-life *(Fig. 3)*. Decomposition of hydroperoxide and malonaldehyde to other lipid oxidation products caused the decline in both PV and TBARS [12]. The results of PV and TBARS were in agreement with earlier reports [20,21].

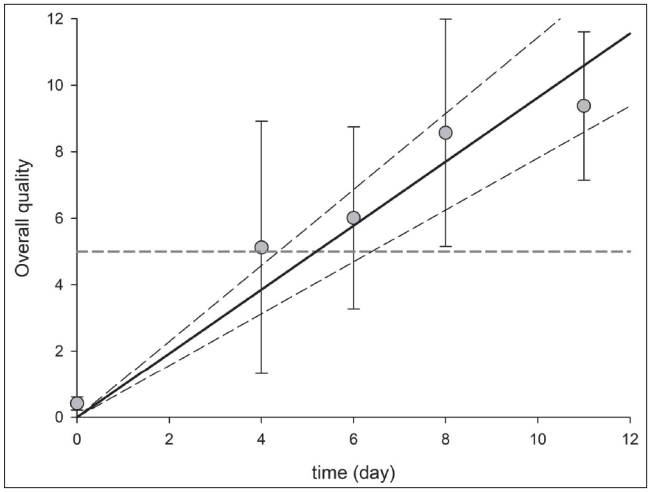

Fig 4. Sensory evaluation of mullet fillets at 0°C with respect to time. Gray circles are the score points; error bars represent standard deviation of the scores from 11 panelists. Solid black line is the model fit [Eq.(3)], dashed black lines are the 95% confidence bands and dashed gray line is the limit for the shelf-life. Original data are from Pilavtepe-Celik et al.[12]

Table 1. *Model equations with respect to analysis (criteria) and model parameters±standard errors obtained by regression together with the goodness-of-fit indices*

Criteria	Eq.	C_0 (mg N/100 g fish)	k (day^{-1})	t_c (day)	R^2	R^2_{adj}	RMSE
Cadaverine	1	–*	6.9±0.4	10.2±0.1	0.9946	0.9936	0.5642
Putriscine	1	–	2.4±0.1	4.7±0.3	0.9921	0.9906	0.7509
TVB-N	2	27.5±0.5	2.6±0.4	7.9±0.7	0.9921	0.9906	0.7509
Sensory	4	–	0.96±0.06	–	0.9190	0.9190	1.0064

** Not a model parameter for the given equation*

Table 2. *Model equations with respect to analysis (criteria) and model parameters±standard errors obtained by regression together with the goodness-of-fit indices*

Criteria	Eq.	C_{max*}	t_{max} (day)	t_{rate} (day)	R^2	R^2_{adj}	RMSE
PV	3	1.1±0.09	9.4±0.4	4.0±0.9	0.9453	0.9180	0.1157
TBARS	3	8.2±0.6	9.8±0.3	4.0±0.9	0.9636	0.9453	0.7272

** Unit of this parameter is mmole CPO/kg fish for PV and mg MDA_{eq}/kg fish for TBARS*

Table 3. *Estimated shelf-life and their calculated 95% confidence intervals of Mullet fillets stored at 0°C*

Criteria	Estimated Shelf-life (day)	95% Interval of the Estimated Shelf-life
Cadaverine	11.7	11.5-11.8
Putriscine	8.9	8.5-9.4
PV	7.5	6.0-ND*
Sensory	5.2	4.4-6.4
TBARS	6.5	5.4-8.8
TVB-N	10.7	10.2-11.3

* Not determined

Subzero temperatures may be used to increase the shelf-life of mullets. Choubert et al.[22] measured TBARS in packed rainbow trout stored at -20°C during a period of 18 months and observed a significant increase in TBARS at the end of first month, but not other changes occurred during the next 5 months. These results revealed that lipid oxidation could be controlled by preservative action of subzero temperatures [23]. The concentration of TBARS in good quality frozen and chilled fish or in fish stored on ice is typically between 5 and 8 mg MDA/kg whereas the levels of 8 mg MDA/kg are generally regarded as the limit of acceptability for most species [24]. In this study, the limit of acceptability was set to 6.5 mg MDA/kg [12]. The duration of shelf-life would have been much higher if the acceptability limit of TBARS had been set to 8 mg MDA/kg.

Not only the overall quality but also flesh color, skin, texture, gaping and odor were also scored during the sensory evaluation [12]. Mathematical modeling, on the other hand, were only applied to overall quality parameter since it was the sole parameter that was used to identify the acceptance and/or rejection point of fish fillets. Sensory analyses were performed with trained panelists as explained above. People in such panels can be considered as "human tools" and are not typically representative for the reaction of consumers since they are trained and selected on their capacity to detect certain changes [25]. Therefore, it was not surprising that the shelf-life based on sensory evaluation was low (5.2 days). Moreover, the uncertainties of the scores were relatively high *(Fig. 4)* revealing the

human subjective inspection [12]. On the other hand, with respect to cadaverine, putrescine and TVB-N much longer shelf-life (8.9 to 11.7 days) were determined compared to PV, TBARS and sensory evaluation *(Table 3)*. Cadaverine and putrescine are good indicators of late spoilage due to late formation [12] hence longer shelf-life were estimated according to these biogenic amines.

It should be noted that according to Eq.(1), cadaverine and putrescine values were assumed to be zero initially ($t = 0$); however, they were not. Nevertheless, this had no consequence and the goodness-of-fit of the model since initially very low (close to zero) values were observed and this was also true for Eq.(3) to estimate PV and TBARS values. It should also be noted that models used in this study were ad hoc or empirical models and had no mechanistic background. In other words, they could all be considered as product oriented yet successful to describe the data since higher R^2 and R^2_{adj}, and lower RMSE values were obtained *(Table 1* and *Table 2)*. Furthermore, empirical models are useful for predicting the shelf-life of fish products [26,27]. It may still be possible to use alternative models with similar goodness-of-fit, but the aim of this study was not to make a comparison between the models.

Predictive modeling can be used to estimate shelf-life of fish products and especially microbial growth models were used for this purpose [28,29]. However, to the best of our knowledge, there are not many modeling studies on sensory and (bio)chemical parameters such as cadaverine, putrescine, PV, TBARS and TVB-N. A notable exception is the work of Calanche et al.[7] where shelf life of sea bream stored in ice were evaluated by using predictive models.

We demonstrated the modeling of mullet fillets with respect to some (bio)chemical parameters and also sensory evaluation to estimate the shelf-life in this study. The procedure presented here can be used for any type of fish with ad hoc models. Of course, more modeling studies based on kinetic mathematical models can be carried out by combining the microbial spoilage with physical and chemical analyses.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available upon request.

Funding Support

The study was not sponsored by any funding sources.

Competing Interest

The authors declare that they have no competing interests.

Author Contributions

Authors contributed equally to this work (design of the study, literature search, data analysis, writing, reviewing) and approved the final manuscript.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 633-639, 2021
DOI: 10.9775/kvfd.2021.26023

RESEARCH ARTICLE

The Effect of Gallic Acid Addition to Tris-Based Extender on Frozen Bull Semen

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Article ID: KVFD-2021-26023 Received: 17.05.2021 Accepted: 17.08.2021 Published Online: 17.08.2021

Abstract

This study aimed to determine the probable protective effect of gallic acid (GA) on the spermatological parameters of frozen-thawed bull semen. Ejaculates were collected from four Holstein bulls. The mixed ejaculate was divided into four equal groups as control (0 µg/mL GA), 50, 100 and 200 µg/mL GA with a Tris-based extender. All extended groups were cooled, equilibrated into a +4°C cold cabinet and loaded into straws. The straws were then frozen and stored in a liquid nitrogen container (-196°C). Subsequently, samples were thawed in a water bath for analyzing motility and kinematic parameters, morphological integrity, DNA damage and biochemical alterations. GA50 (28.76±0.51%) and GA100 (29.32±0.31%) had improved progressive motility in comparison to the other groups (P<0.05). The highest total motility (69.71±0.52%) was detected in GA100 (P<0.05). Besides, the lowest DNA damage and total abnormality values were detected in the GA100 group (P<0.05). GA100 had the highest total antioxidant capacity, as well as the lowest malondialdehyde (MDA) level (P<0.05). In conclusion, GA 50 and 100 µg/mL protects the progressive motility, morphological and DNA integrity by improving the total antioxidant status from the harmful effects of the freezing and thawing protocol.

Keywords: *Antioxidant, Bull semen, DNA damage, Gallic acid*

Boğa Sperması Dondurulması Üzerine Tris Sperma Sulandırıcısına İlave Edilen Gallik Asidin Etkisi

Öz

Bu çalışmada, gallik asitin (GA) dondurulmuş çözdürülmüş boğa sperması spermatolojik parametreler üzerine olası koruyucu etkisinin belirlenmesi amaçlandı. Ejakulatlar dört adet Siyah ala boğadan alındı. Birleştirilen ejekulatlar kontrol (0 µg/mL GA), 50, 100 ve 200 µg/mL GA olmak üzere Tris bazlı sulandırıcı ile dört eşit gruba ayrıldı, Sulandırılan gruplar +4°C sıcaklığındaki soğuk kabinde soğutularak ekilibre edildi, payetlendi ve dondurularak sıvı azot tankında (-196°C) depo edildi. Daha sonra örnekler motilite ve kinematik parametreler, morfolojik bütünlük, DNA hasarı ve biyokimyasal analizler için çözdürüldü. Diğer gruplar ile karşılaştırıldığında GA 50 (%28.76±0.51) ve GA 100 (%29.32±0.31) progresif motiliteyi artırdı (P<0.05). En yüksek total motilite %69.71±0.52 ile GA100 grubunda tespit edildi (P<0.05). Bunun yanı sıra, en düşük DNA hasarı ve total anormal spermatozoa oranı GA 100 grubunda belirlendi (P<0.05). GA100 grubunda en yüksek total antioksidan kapasitenin yanında en düşük malondialdehit seviyesi (MDA) tespit edildi (P<0.05). Sonuç olarak, 50 ve 100 µg/mL dozlarındaki GA'nın ilavesi progresif motilite, total motilite, morfolojik ve DNA bütünlüğünü total antioksidan kapasiteyi artırarak dondurma ve çözdürmenin zararlı etkisinden koruduğu belirlendi.

Anahtar sözcükler: *Antioksidan, Boğa sperması, DNA hasarı, Gallik asit*

How to cite this article?

Güngör Ş, İnanç ME, Yeni D, Avdatek F, Türkmen R, Taşdemir U: The effect of gallic acid addition to tris-based extender on frozen bull semen. *Kafkas Univ Vet Fak Derg,* 27 (5): 633-639, 2021.
DOI: 10.9775/kvfd.2021.26023

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Introduction

Semen freezing and artificial insemination (AI) have a potential to increase cattle production. Through the use of AI with frozen semen, semen from high-quality bulls may be used to inseminate thousands of cattle around the world [1]. The frozen semen use is the most suitable method for controlling diseases in high-yielding growing areas, as well as transfer of genetic information to other generations and spreading superior productive breeds [2-4]. Besides, semen freezing technology is very important for the success of AI, which has become the prior technique of breeding in the cattle industry [5]. In addition to these positive effects, the semen freezing protocol induces some irreversible damage to spermatozoa and reduces the live spermatozoa rate, as well as increasing aneuploidy and DNA damage [6-9]. The live spermatozoa rate and DNA integrity have a crucial role in sperm survival in frozen-thawed semen during AI for transmission of genetic material and early embryonic development [10,11]. During the freezing of sperm, the increase in reactive oxygen species (ROS) results in an inability to perform cell functions, and consequently, a gradual decrease in morphological integrity and motility reduces fertilization ability with sperm apoptosis [12]. There is a strong relationship between reduced sperm motility and oxidative stress by ROS [13]. Malondialdehyde (MDA) markers are commonly used to detect oxidative stress [14]. Antioxidant compounds control the effects of ROS and protect spermatozoa against oxidative stress. Non-enzymatic antioxidants also decrease ROS and indirectly protect the plasma membrane from lipid peroxidation. These oxidations and antioxidation levels can be determined based on MDA, which is formed upon peroxidation of lipids [15]. Due to these problems that arise during freezing, researchers have focused on semen diluents to develop an optimum diluent and freezing protocol by introducing new additives. The triphenolic compound gallic acid (GA) (3,4,5-trihydroxy benzoic acid) exists in some plants with organic acids such as lemons, grape seeds, bananas, carobs, sumac, apples, strawberries, gallnuts, and pineapples [16-18]. It is produced as a secondary metabolite pathway shikimic acid in plants [16]. Researchers have reported that GA has antioxidant, antiviral, anti fungal, anti-inflammatory, antitumor and antimutagenic effects [18-21]. Bello and Idris [22] reported that GA is an anti oxidant, and it is formed sometimes as a part of a free molecule or sometimes as a part of a tannin molecule.

This study aimed to determine the effects of GA on the *in vitro* spermatological parameters of motility and kinetic parameters, DNA integrity, morphological integrity and certain biochemical alterations of frozen-thawed bull semen.

Materials and Methods

Ethical Approval

Ethical permission for the study was given by the Afyon Kocatepe University, School of Veterinary Medicine, Animal Experiments Ethics Committee (with the authorization number of 49533702/29).

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co., unless otherwise stated.

Animals and Study Design

Forty ejaculates were collected at different times during the same seasonal period (from January to March) with an artificial vagina from four 2-4-year-old healthy Holstein bulls that were raised in the Sultansuyu Agriculture Business (Sultansuyu, Malatya, Turkey) affiliated with the General Directorate of Agricultural Enterprises. After collection, the semen was left in a water bath at 37°C, and the mass activity of the fresh semen was scored on a hot plate [on a scale of 1 to 5]. Semen volume was recorded with a graded collection tube. The concentration (mL/spermatozoa) of semen was recorded with the photometrical method (Minitube GmbH, Tiefenbach, Germany). The ejaculates with satisfactory characteristics (mass activity ≥3, concentration ≥800×10^6/mL and volume ≥5 mL) were used. The semen was mixed for minimizing differences from the bulls and volume.

Semen Processing and Freezing

The main Tris-based extender (248.22 mM Tris [T1503], 85.36 mM citric acid [C0759], 69.93 mM fructose [F0127], egg yolk 20% v/v and 6% glycerol, 310 mOsm) was prepared with the protocol described by Taşdemir et al.[23]. A 10 mg of GA [G7384] was dissolved with 1 mL of ethanol (Merck, 99%), and the GA stock solution was prepared. The mixed semen was divided into four groups and diluted with the Tris extender containing 0 µg/mL GA (control), 50 µg/mL GA (GA50), 100 µg/mL GA (GA100) and 200 µg/mL GA (GA200). All extended groups were cooled slowly in a water bath (22°C) and equilibrated for 3 h into a +4°C cold cabinet. After equilibration, the diluted semen groups were loaded into straws (0.25 mL) with 16×10^6 sperm cells in each straw, and then, frozen by using automated semen freezing machine (SY LAB Gerate GmbH, Neupurkersdorf, Austria) based on the guidelines reported by Avdatek et al.[24]. After storage (at least 3 mounts), the sample was thawed in a water bath (37°C/30 s for spermatological evaluations.

Spermatozoa Motility and Motility Motion Characters

A computer assisted semen analyzer (SCA, Sperm Class Analyzer, Microptics, Spain) was used to analyze various kinetic parameters and sperm motility after thawing (37°C, 30 s). The sperm motility properties were set as fast (>80 µm/s), medium (>60 µm/s), slow (>20 µm/s) and static. The semen sample (5 µL) was placed onto the slide, covered with a coverslip and analyzed with a 10× lens on

a preheated microscope stage (37°C). Total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm/s), beat-cross frequency (BCF, Hz), linearity (LIN%) [(VSL/VCL)x100], wobble (WOB%) [(VAP/VCL)x100], straightness (STR%) [(VSL/VAP)x100], and hyperactivation [(VCL/VSL)xALH] were determined. A total of 200-400 spermatozoa per sample were evaluated in five microscopic zones [25].

Abnormal Spermatozoa Rate

The abnormal spermatozoa rate was determined via the fixation method using a Hancock solution under a phase-contrast microscope at 1000x magnification. The Hancock's fixation solution was prepared to contain a saline solution (150 mL), buffer solution (150 mL), distilled water (500 mL) and formalin (62.5 mL of 37%). For spermatozoa fixation, a 1/200 working solution (semen/Hancock solution) was prepared. A 6 µL of the working solution was placed on a slide and covered with a cover slide, and abnormal spermatozoa were determined by counting 300 spermatozoa in a 100x phase-contrast microscope through an immersion lens. The percentages of the normal and abnormal spermatozoa (head, midpiece and tail anomalies) were recorded [26].

Sperm DNA Integrity

Sperm chromatin damage was assessed using the alkaline single-cell gel electrophoresis (COMET Assay) method. The samples (slides) were examined under a fluorescent microscope (Olympus CX31), and the resultant images were scored using the Comet Score software (TriTek, V. 1.5). DNA integrity determination was held on basic protocol; eliminating captures not corresponding to Comets (i.e., debris); removing overlapping Comets; or correcting the head and tail detection for those Comets that were misanalysed. When the final number of correctly analyzed Comets was less than 50, more images from slides were captured and the process was repeated until obtaining at least 50 correctly analyzed images. Tail DNA, tail moment and tail length are relative measurement of the amount of DNA in the Comet tail in relation to the total DNA amount, expressed in percentage and calculated as: (tail intensity)/(comet intensity). Tail length was calculated as: (tail main intensity + head mean intensity)/2. Tail moment takes into account the amount of tail DNA, but also the differences of optical intensities between the Comet head and the Comet tail. This parameter was calculated as: (tail mean intensity-head mean intensity)xtail DNA/100. In every specimen, a total of two hundred spermatozoa observed in six different zones were assessed [27].

Biochemical Alterations

Total antioxidant capacities (TAC) were calculated according to Erel's procedure, spectrophotometrically at 660 nm [28]. A TAC Rel Assay brand kit (Rel Assay Diagnostics, Gaziantep, Turkey) was used. The discoloration of antioxidant molecules was used to make the calculation. As a calibration, Trolox was used. The results are presented as mmol Trolox Eq/L. The reference range for human serum is given by the manufacturer as 1.20-1.50 mmol/L, no data being available for seminal plasma. Measurements in duplicate were used to calculate intra-assay variability.

A MDA commercial kit (MDA586; OxisResearch, Portland, OR, USA) was used to assess MDA as a lipid peroxidation marker. The reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI), with MDA at 45°C was used to make the measurement. A spectrophotometer was used to calculate the absorbance at 586 nm (Shimadzu UV-1601). MDA values were determined in µmol/mL [28]. The absorbance at 586 nm is a linear function of the MDA concentration over the range from 0.5 µM to 20 µM.

Glutathione peroxidase (GPx) enzyme activity in the semen was assessed using an Oxis Research test kit (Bioxytech® GPx340™, Portland, Oregon, USA). The GPx-340™ assay is an indirect indicator of cellular glutathione peroxidase (cGPx) activity. To test for cGPx, the sample was mixed with glutathione, glutathione reductase and NADPH in a solution. The tertbutyl hydroperoxide enzyme reaction was started by adding the substrate. The absorbance at 340 nm was measured every 15 sec for 3 min. The GPx activity in the sample was directly proportional to the rate of the decrease in absorbance at 340 nm. The findings are expressed in mU/mL [28]. While samples with a change in absorbance of less than 0.035 A340/min can be accurately assayed if the protocol is modified (longer reaction times), OXIS recommends that the researcher obtain values from 0.035 to 0.15 A340/min using the stated protocol. This corresponds to approximately 5.6 to 24 mU/mL enzyme activity.

Statistical Analysis

Before the significance tests, the obtained data were tested for normal distribution by Shapiro Wilk test. The homogeneity of the variances was examined with Levene's test. The statistical analysis of the difference between the variables was achieved with analysis of variance (ANOVA). Tukey's test was used to evaluate the differences between the groups. Descriptive statistics for each variable were calculated and are presented as "Mean ± Standard Mean Deviation" (Mean ± SD). All statistical analyses were interpreted with a minimum error of 5%. Using the SPSS 22.0 package program, $P<0.05$ was considered statistically significant.

Results

The results on semen CASA motility and kinetic parameters are given in *Table 1*. The GA50 (28.76±0.51%) and GA100 (29.32±0.31%) groups had improved motility in comparison to the other groups ($P<0.05$). Additionally,

Table 1. *CASA motility and kinetic parameters values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Progressive motility (%)	26.61±0.65^{a}	28.76±0.51^{b}	29.32±0.31^{b}	27.07±0.41^{a}	*
Total motility (%)	66.25±0.39^{b}	66.13±0.51^{b}	69.71±0.52^{a}	64.90±0.52^{b}	*
VAP (µm/s)	50.54±0.53^{b}	49.02±0.37^{ab}	47.83±0.77^{a}	52.60±0.66^{c}	*
VSL (µm/s)	35.74±0.28^{a}	39.82±0.40^{c}	37.77±0.26^{b}	36.94±0.51^{b}	*
VCL (µm/s)	80.54±0.54^{a}	86.77±0.89^{b}	86.37±0.74^{b}	85.64±1.19^{b}	*
ALH (µm/s)	3.89±0.09^{a}	3.89±0.06^{a}	4.15± 0.05^{b}	4.18±0.04^{b}	*
BCF (Hz)	12.27±0.29	12.82±0.23	12.42±0.22	12.34±0.34	-
LIN (%)	44.30±0.46^{ab}	45.87±0.88^{b}	43.70±0.81^{ab}	42.93±0.73^{a}	*
STR (%)	74.56±0.96	75.73±0.83	74.61±0.79	74.83±0.51	-
WOB (µm/s)	59.38±0.65^{ab}	58.51±0.89^{ab}	60.56±0.68^{b}	57.35±0.90^{a}	*
Hyperactivity (%)	27.31±2.20	29.66±2.93	30.64±1.89	27.47±0.62	-

^{a,b,c,d} *Different superscripts within the same row demonstrate significant differences (*P<0.05); - No significant difference (P>0.05)*

Table 2. *Spermatozoa abnormality values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Head (%)	8.90±0.39^{b}	7.88±0.70^{ab}	7.11±0.26^{a}	7.91±0.49^{ab}	*
Mid-piece (%)	10.54±0.33	10.17±0.17	9.68±0.50	10.22±0.72	-
Tail (%)	6.92±0.28	6.45±0.30	6.24±0.16	6.56±0.11	-
Total (%)	26.36±0.94^{c}	24.51±0.51^{b}	23.03±0.44^{a}	24.69±0.31^{b}	*

^{a,b,c,d} *Different superscripts within the same row demonstrate significant differences (*P<0.05); - No significant difference (P>0.05)*

Table 3. *Chromatin damage values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Tail lenght (µm/s)	21.16±0.29^{c}	14.81±0.18^{b}	11.95±0.29^{a}	15.6±0.30^{b}	*
Tail DNA (%)	34.15±0.46^{d}	21.96±0.30^{b}	19.86±0.97^{a}	27.78±0.41^{c}	*
Tail moment (µm/s)	20.03±0.20^{d}	10.96±0.26^{b}	7.20±0.10^{a}	17.25±0.37^{c}	*

^{a,b,c,d} *Different superscripts within the same row demonstrate significant differences (*P<0.05)*

the highest total motility (69.71±0.52%) was detected in the GA100 group (P<0.05). The spermatozoa were evaluated in terms of kinetic properties, where the highest VAP (µm/s) was detected in GA200, while the lowest VSL (µm/s), VCL (µm/s) and ALH were detected in the control group (P<0.05). There were differences between the GA50 (45.87±0.88%) and GA200 (42.93±0.73%) groups in terms of their LIN parameters (P<0.05). There were no differences among the groups based on their BCF (Hz) and hyperactivity (%) values (P>0.05).

The results of the morphological evaluation (spermatozoa abnormality) are given in *Table 2*. There were no significant differences between the groups based on their midpiece and tail abnormality rates (P>0.05). The lowest head (7.11±0.26%) and total abnormality (23.03±0.44%) rates were in the GA100 group (P<0.05).

DNA damage was significantly different among the treatment groups (P<0.05) as shown in *Table 3*. The lowest comet evaluation parameters of tail length (µm/s), tail DNA (%) and tail moment (%) were detected in GA100. In control group chromatin damage values [tail length (µm/s), tail DNA (%) and tail moment (%)] were higher than the treatment groups (P<0.05). The biochemical alterations of the frozen-thawed bull semen parameters are shown in *Table 4*. The GA100 groups displayed the highest TAC (0.81±0.03 Trolox equiv./L) and GPx activity (11.14±0.33 mU/mL) (P<0.05), as well as the lowest MDA level (7.05±0.38 µmol/mL) (P<0.05). Highest MDA level and lowest GPx activity were shown in the control group (P<0.05).

Discussion

At artificial insemination and *in vitro* fertilization organizations/procedure/application, spermatozoa motility is great importance for successful fertilization [29]. During fertilization, it has been shown that the spermatozoa are transported to the fertilization area to combine with the ovum [30]. Frozen

Table 4. *Total antioxidant capacities (TAC), malondialdehyde (MDA) levels and glutathione peroxidase (GPx) activities in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
TAC (mmol Trolox Eq/L)	0.36±0.01[a]	0.40±0.02[a]	0.81±0.03[d]	0.59±0.03[c]	*
MDA (µmol/mL)	12.92±0.36[c]	7.99±0.43[a]	7.05±0.38[a]	9.30±0.15[b]	*
GPx (mU/mL)	8.62±0.10[a]	9.66±0.13[b]	11.14±0.33[c]	9.86±0.21[b]	*

*[a,b,c,d] Different superscripts within the same row demonstrate significant differences (*P<0.05); - No significant difference(P>0.05)*

thawed bull semen is more easily peroxidized than fresh sperm. Additionally, intracellular antioxidant capacity in sperm decreases following the freeze-thawing process [31]. The axosome and associated dense fibers of the middle pieces in spermatozoa are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility. In this study, the highest total motility values were detected in the GA100 group (P<0.05). In comparison to the other groups, GA100 and GA50 had improved progressive motility values (P<0.05). The positive effects of GA addition (except for the GA200 group) on progressive motility were supported by other researchers. Gungor et al.[32] supplemented GA into tris a ram semen extender and observed improved levels of post-thawing motility. Tris extender with GA was seen to improve viability and plasma membrane integrity in ram semen [33]. Although the species whose sperms were frozen were different, it was interpreted that this positive effect of GA was due to its cryoprotective effect on spermatozoa in harmony with other components added to the diluent. Besides motility, CASA kinetic parameters are important for the evaluation of individual spermatozoa movements. Many researchers have investigated the relationship among kinetic parameters in bull spermatozoa and conducted in vitro oocyte binding ability and fertilization studies in the field [34,35]. Amann [36] examined motility kinetic parameters in frozen-thawed bull semen, and they determined the VCL, VSL, LIN, ALH and BCF parameters to be associated with the fertility index. Nagy et al.[37] found that VCL, VSL and VAP values among velocity parameters are more useful for reflecting the results of AI. In this study, there was no significant difference among the groups in terms of their spermatozoa kinetic parameters compared control except for VAP, ALH, VSL and VCL. Incompatibility with this study, Daghigh et al.[38] was detected an ameliorating effect on the VSL, VCL and progressive motility values of frozen-thawed bull semen by supplementation of Origanum vulgare, which contains natural antioxidants and high levels of phenolic compounds including gallic acid. These supporting results were also in line with the reports of Osawa [39], who discovered that GA inhibits the adverse effects of free radicals, metal ions and hydroxyl groups by binding them. Lozano et al.[40] reported that the pro-oxidant actions of polyphenols (PP), flavonoids, anthocyanin's and carotenoids are typically catalyzed by transition metals such as Cu and Fe within cells under certain O_2 and pH conditions. GA can act as antioxidant/pro-oxidant reactions associated with efficient electron transfer capacity. Thus, it inhibits the uncontrolled ROS production to prevent the cell damage [41]. Moreover, a positive correlation was found between this compound and the LIN parameter in a ram semen cervical mucus test [30]. However, the results of the treatment and control groups were not different in the aforementioned study.

There were no significant differences among the groups in terms of their midpiece and tail abnormality rates (P>0.05). The lowest head and total abnormality rates were in the GA100 group (P<0.05). This result was also supported by the lowest DNA damage in this group (P<0.05). Majorly morphologically abnormal spermatozoa have been associated with decreased fertility, and morphological evaluation is used to provide an indication of the potential fertility of spermatozoa in bulls, whereas sperm DNA damage has an adverse effect on embryo development and subsequently fertility [42]. Consistent with this study, Mehraban et al.[18] identified the protective effect of GA on cyclophosphamide (chemotherapy drugs) toxicity in terms of not only morphological and DNA integrity but also viability and concentration. GA was also found to reduce abnormal spermatozoa rates in mice sperm induced with exposure to opioid drugs with analgesic properties (morphine) [43]. In the present study, the lowest DNA damage was observed in the GA100 group. In support of our findings, Wen Weng et al.[44] reported that GA alleviated DNA damage by DNA repair-associated protein expression in the SCC-4 cell line, namely human oral cancer cells. This result showed that GA stimulates cytotoxic effects on human cancer cells by cell-cycle arrest and induction of apoptosis. Erol et al.[45] found that GA significantly reduced both mitochondrial DNA and nuclear DNA damages that were induced by H_2O_2 exposure. When GA is used at the optimum doses, it is thought that it improves DNA integrity and repairs morphological abnormalities thanks to its antioxidant and anti-mutagenic effects.

Cryopreservation reduces the functional and structural integrity of bull spermatozoa, and is associated with ROS production. Oxidative stress during freezing of semen can induce functional and structural damage to spermatozoa involving ROS-mediated pathways [7]. Although bull semen has a natural defense system against the ROS, it is considered insufficient in protecting spermatozoa under cryopreservation mediated stress [46]. Hence, GA's mitochondrial PP acts might be occurring combined effect of antiproliferative mechanism and antioxidant activity

from more than one triggered mechanism. Beyond their antioxidant activity, some PP may decrease mitochondrial membrane fluidity or have a molecular mechanism related to: mimicking of the Bcl-2 homology-3 (BH3) domains, hexokinase inhibition and thiol redox inhibition [47]. Nevertheless, despite the fact that not all PP have the same properties and mitochondrial-related mechanisms, all of them have ROS-scavenging actions either at the ROS-removing or ROS-formation levels [48]. Also, PP cellular environment and concentration may influence these actions, and whether PP acts as pro-oxidant molecules. Although the selective antiproliferative effects of pro-oxidant PP are not fully known, it is attributed to the formation of an unstable redox complex with a metal cation supported by an unstable radical aroxyl or pro-oxidant PP, as metal ions catalyze the formation of ROS via Fenton or Fenton-like reactions [49]. Therefore, the addition to semen extenders of suitable antioxidants is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa [50]. GPx and TAC indicative activity of antioxidant levels. In this study, the highest TAC and GPx activity were detected in the GA100 group ($P<0.05$), which also showed the lowest MDA (not different from GA50) level. In line with this study, Jofre et al.[51] stored boar semen with different concentrations of Murtilla (Ugni molinae Turcz (average phenolic content includes gallic acid) extract at 17°C for 168 h. The long-term analyses showed that the Murtilla extract had a protective role on semen motility decay and reduced ROS and membrane damage. GA is known to effects inflammatory bioactivities and exhibit antioxidant properties [19]. That could explain all these positive effects of GA by protection against oxidative stress.

In conclusion, it was determined that supplementation of GA by 50 and 100 µg/mL into tris-egg yolk semen extender protected progressive motility, total spermatozoa abnormality and DNA integrity from the harmful effects of the freezing and thawing protocol by the improved total antioxidant status.

Acknowledgement

This study was supported by Sultansuyu Agriculture Business (Sultansuyu, Malatya, Turkey) affiliated with the General Directorate of Agricultural Enterprises.

Conflict of Interest

There is no conflict to interest.

Author Contributions

ŞG examined abnormal spermatozoa rate, did the statistical analysis and prepared original manuscript draft, MEİ examined abnormal spermatozoa rate, determined semen motility and motility kinetic parameters, prepared the original manuscript draft, DY and FA specified DNA damage, RT analyzed biochemical alterations, UT designed the study, froze the semen, determined motility and motility kinetic parameters and edited the manuscript.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 641-648, 2021
DOI: 10.9775/kvfd.2021.26134

RESEARCH ARTICLE

Effect of Pasture Versus Indoor Feeding on Milk Microbiota of Goats

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Article ID: KVFD-2021-26134 Received: 11.06.2021 Accepted: 10.09.2021 Published Online: 10.09.2021

Abstract

The microbial profile of milk can influence the quality of raw milk and milk products. To investigate whether the feeding styles of goats affected their milk microbiota profile, two local goat farms with different feeding styles (pasture and indoor feeding) were selected. Milk samples contained 10 colostrum samples (5 from pasture-raised goats and 5 from indoor-fed goats) and 12 mature milk samples (7 from pasture-raised goats and 5 from indoor-fed goats) were collected. 16S rDNA sequences of these samples were amplified and further subjected to alpha- and beta-diversity analysis, principal coordinates analysis (PCoA), linear discriminant analysis effect size (LEfSe) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The result showed that pasture-raised goats showed higher milk microbial abundance and diversity than indoor-fed goats. Specifically, *Propionibacterium, Weeksellaceae, Lactobacillus, Cloacibacterium,* and *Yersinia* were enriched in colostrum and *Betaproteobacteria, Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas,* and *Gluconacetobacter* were enriched in mature milk of pasture-raised goats. In addition, the functional metabolic genes of the milk microbiota differed significantly in goats of these two farms. Altogether, the present study analyzed the microbiota of colostrum and mature milk of goats and suggested that feeding style could profoundly affect the composition of milk microbiota.

Keywords: *Goat, Pasture, Indoor feeding, Feeding style, Milk microbiota*

Meraya Dayalı Beslemeye Karşı Kapalı Beslemenin Keçilerde Süt Mikrobiyotası Üzerine Etkisi

Öz

Sütün mikrobiyal profili, çiğ süt ve süt ürünlerinin kalitesini etkileyebilir. Keçilerin beslenme biçimlerinin süt mikrobiyota profilleri üzerine etkilerini araştırmak için farklı besleme özelliğine (mera ve kapalı besleme) sahip iki yerel keçi çiftliği seçildi. On adet kolostrum örneği (5'i merada beslenen ve 5'i kapalı alanda beslenen keçilerden) ve 12 adet ergin hayvana ait süt örneği (7'si merada beslenen ve 5'i kapalı alanda beslenen keçilerden) toplandı. Bu örnekler, 16S rDNA dizilerinin amplifikasyonu sonrasında alfa ve beta çeşitlilik analizine, temel koordinat analizine (PCoA), doğrusal diskriminant analizi etki büyüklüğü (LEfSe) analizine ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) yolak analizine tabi tutuldu. Merada beslenen keçilerin, kapalı alanda beslenenlere göre süt mikrobiyotasının daha bol olduğu ve daha fazla çeşitlilik gösterdiği saptandı. Meraya bağlı beslenen keçilerde kolostrumda özellikle *Propionibacterium, Weeksellaceae, Lactobacillus, Cloacibacterium* ve *Yersinia* türleri, ergin hayvan sütlerinde ise *Betaproteobacteria, Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas* ve *Gluconacetobacter* türleri daha fazlaydı. Ayrıca, bu iki çiftliğin keçilerinde süt mikrobiyotasının fonksiyonel metabolik genleri önemli ölçüde farklılık gösterdi. Bu çalışmada, keçilerin kolostrum ve ergin sütünün mikrobiyotası analiz edilmiş ve besleme tarzının süt mikrobiyotasının bileşimini ciddi şekilde etkileyebileceği öne sürülmüştür.

Anahtar sözcükler: *Keçi, Mera, Kapalı besleme, Besleme şekli, Süt mikrobiyotası*

INTRODUCTION

It is widely believed that the mammary gland is a sterile organ. However, recent progress in culture-independent technologies and data analysis methods has challenged this long-held notion by showing that there exists a complex microbial community in the milk [1-3]. In fact, the presence of milk microbiota has been documented in

How to cite this article?

Jing T, Yao X, Ji T, Dong J, Chen X, Chen F, Feng H, Wang T, Zhao H, Chen D, Ma W: Effect of pasture versus indoor feeding on milk microbiota of goats. *Kafkas Univ Vet Fak Derg,* 27 (5): 641-648, 2021.
DOI: 10.9775/kvfd.2021.26134

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many hosts including humans [4], cows [5], goats [1], sheep [6] and water buffalos [7], with most studies concentrated on humans and cows.

Mounting evidence shows that milk microbiota has a great impact on the seeding of an infant's gut microbiota. For example, Jost et al.[8] found several gut-associated obligate anaerobic genera and members of the *Clostridia* were shared between breastmilk and the related neonatal feces over the first month of life. Supporting this finding, Murphy et al.[9] found identical strains of bacteria could be isolated from the breastmilk and feces of the infant, with shared genera between breastmilk and neonatal feces accounted for 70~88% of infant fecal microbiota. In consistent with this finding, Williams et al.[10] found that the milk microbiota of breastfeeding mothers contributed approximately 4.9% to the gastrointestinal bacterial communities of their infants.

Recent studies also suggested that the microbial profile of the milk is associated with the health condition of the lactating animal and hence affecting the quality of subsequent dairy products. Mastitis is a complex disease of dairy animals and has brought great economic losses to dairy industries worldwide [11]. Recent data suggest that mastitis is not merely caused by pathogen infections, but also by the consequence of intramammary microbiota dysbiosis. For example, the development of bovine mastitis was associated with decreased bacteria diversity and increased abundance of opportunistic pathogens, a phenomenon also observed in humans [2,12-14]. In water buffalos, the development of subclinical mastitis was associated with the decrease in the relative abundance of genera *Psychrobacter*, *SMB53* and *Solibacillus* in the milk [15]. Accordingly, milk microbiota profile was suggested to serve as an effective approach to distinguish cows with or without mastitis [16,17].

The milk microbiota is not constantly stable but is a dynamic ecosystem affected by various factors. It is commonly recognized that both host and environmental factors influence milk microbiota composition. For host factors, the genetic traits, physiological status and anatomical characteristics of the udder and lactation stage are suggested to affect the milk microbiota of cows and sows [18-20]. In humans, delivery mode is also an important determinant of milk microbiota composition, with higher bacterial diversity found in women with vaginal delivery compared with those who deliver through cesarean section [4,21]. For environmental factors, the farming environment plays an important role in affecting milk microbiota. For example, Metzger and colleagues found that the overall milk bacterial community was affected by bedding types of cows, although the sample size is relatively small in this study [22]. In addition, feeding style was shown to correlate with milk microbiota diversity of cows. For example, high-concentrate diet feeding in Holstein dairy cows could result in elevated mastitis-causing bacteria in the milk [23]. Other studies also showed that total mixed ration with artemisinin or lactic acid supplementation could remarkably affect the composition of milk microbiota of dairy cows [24,25]. Pasture and indoor feeding are two principle feeding styles of dairy goats in China. However, it is unclear whether these feeding styles have an effect on milk microbiota composition.

The aim of this study was to investigate whether feeding styles of goats affected their milk microbiota profile. For this purpose, outdoor pasture-grazing or indoor-fed goats were selected and 16s rDNA of their milk microbiota was sequenced and analyzed. The results showed that feeding style could greatly affect the composition and functional metabolic genes of milk microbiota.

Material and Methods

Description of Samples

A total of 22 goats (female; 1-year-old) were used in this study. The milk samples contained 10 colostrum samples (5 from pasture-raised goats; 5 from indoor-fed goats) and 12 mature milk samples (7 from pasture-raised goats; 5 from indoor-fed goats). 16S rDNA sequences of these samples were amplified and studied. All screened goats were healthy.

DNA Extraction

For each milk sample, microbial genomic 16S rDNA was extracted with a milk DNA kit from Omega Bio-tek (Norcross, GA, USA). The DNA quality was examined through 2% agarose gel electrophoresis. The concentration was measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were kept at -20°C before further analysis. The analyzers were blinded to all testing samples when the rDNA was further analyzed.

PCR Amplification of 16S rDNA

The illumine sequencing library was constructed with the V3-V4 region of 16S rDNA, which was amplified with the conserved primers 805R (5′-GACT ACHVGGGTATCTAATCC-3′) and 341F (5′-CCTACGGGNGGCWGCAG-3′). Then the different identifier codes at each primer were added. For PCR analysis, a 50 μL reaction system containing 25 μL of 2 × Phanta Max Master Mix (Vazyme, Jiangsu, China), 16 μL of ddH_2O, 10 mM each primer, and 5 μL of DNA template was set. The PCR program was first denaturation at 95°C with 10 cycles for 30 s, then annealing at 55°C for 30 s and extension at 72°C for 45 s, at last with an elongation condition at 72°C for 5 min. A Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Waltham, MA, USA) was used to estimate the quality of the PCR products for library preparation. Barcoded samples were combined with the same concentrations. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure

the library concentration following the elution with Tris-HCl (pH 8.5). The samples were then sequenced in MiSeq Reagent Kit V3 (600-cycle) on a PE250 v3 instrument of MiSeq Plateform (Illumina, San Diego, CA, USA).

Bioinformatics and Statistical Analysis

All sequences in this study have been deposited to National Center for Biotechnology Information (NCBI) database with the accession number XC190402. The QIIME (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/) was used to manage the raw reads and FLASH v1.2.7 was performed to assemble the paired reads. Meanwhile, QIIME was performed to screen and analyze the sample sequences. Operational taxonomic units (OTUs) were acquired by UPARSE 7.0 with 97% identity threshold. After that, the whole OTUs were grouped to specific taxonomic levels through Ribosomal Database Project (RDP) classifier 2.2. R program Venn-Diagram package was performed to make the Venn diagram corresponding to the OTU information. The phylogenetic tree was acquired following the sequences alignment by MEGA 5.2. MOTHUR was used to measure the microbial community diversity, alpha diversity. To estimate the similarities of these samples, Bray-Curtis distance was measured by R program vegan package. OIIME was also used to obtain phylogenetic beta diversity. According to Bray-Curtis distance, principal coordinate analysis (PCoA) by R program was performed. A statistical significance test was conducted by PERMANOVA and student's *t* test.

Results

Description of the Sequencing Data

Milk was collected from pasture-raised or indoor-fed goats. We retrieved 709.380 raw reads from the sequencing platform and filtered 111.176 reads with an average of 417 bp in length for further analysis.

Milk Microbiota is Affected by Different Feeding Styles

After filtering the raw sequences, 111.176 high-quality available reads were left for further analysis. Based on 97% sequence similarity, 2.339 and 2.078 OTUs were obtained from colostrum samples of pasture-raised (P) and indoor-fed goats (I), respectively. Meanwhile, 2.363 and 2.262 OTUs were obtained from mature milk samples of pasture-raised and indoor-fed goats, respectively *(Fig. 1-A)*. A total of 5.960 OTUs were detected from all milk samples, of which 1653 and 1701 were core OTUs of colostrum and mature milk, respectively *(Fig. 1-A)*. The core OTUs composed approximately 56.28% of the whole OTUs. As for colostrum, 686 and 425 OTUs were uniquely existed in pasturing goats and indoor-fed goats, respectively *(Fig. 1-A)*. For mature milk, 934 and 561 OTUs were uniquely existed in pasture-raised and indoor-fed goats, respectively *(Fig. 1-A)*. Interestingly, the observed OTUs in pasture-raised goats were higher than that in indoor-fed goats both for the colostrum and mature milk *(Fig. 1-A)*.

Furthermore, alpha and beta diversities were measured to estimate the quality of these sequencing data. Alpha diversity index of colostrum diverged significantly between these differently-fed goats *(Fig. 1-B)*. Although there was a similar trend for that of mature milk, the difference was not statistically significant *(Fig. 1-B)*. The Shannon-Wiener values of colostrum from pasture-raised and indoor-fed goats were 4.45 and 3.97, respectively *(Fig. 1-B)*. And the values for mature milk were 4.37 and 4.11, respectively *(Fig. 1-B)*. Within each group, Shannon index showed that the diversity of goat milk microbial population in pasture-raised goats was higher than that in indoor goats, although

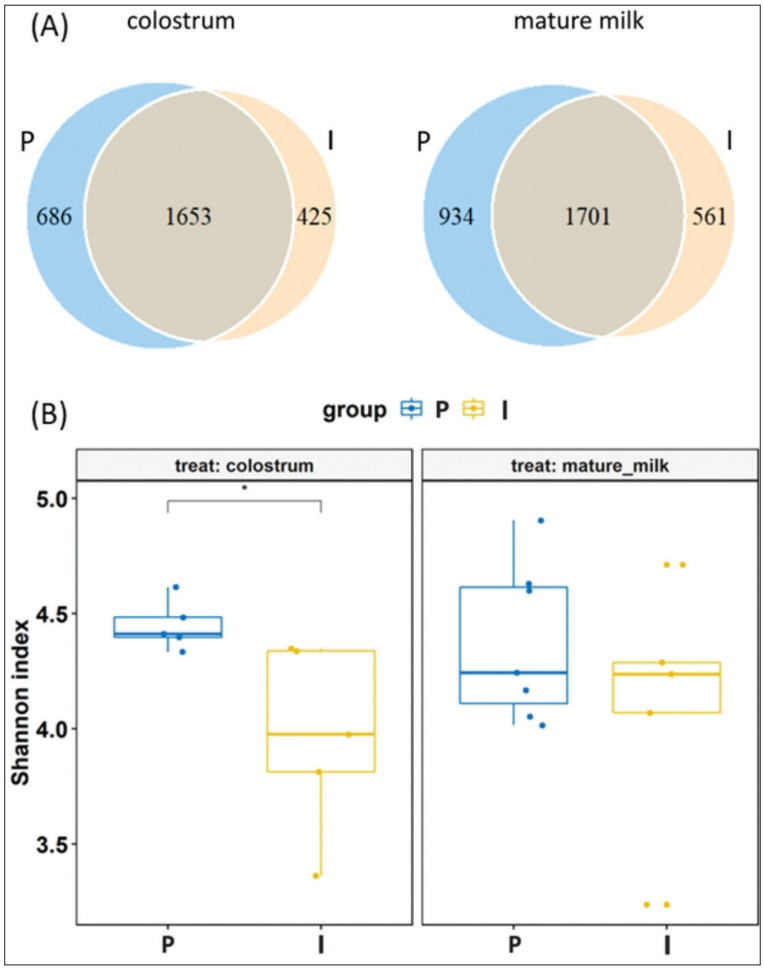

Fig 1. The community composition and microbial diversity index analysis of colostrum and mature milk. (A) Venn diagram showing overlap in OTUs of differential abundance in pasture-raised (P) and indoor-fed goats (I). (B) Shannon index analysis of colostrum and mature milk. Asterisks represent statistical significance (*P<0.05, ** P<0.01, *** P<0.001)

the difference was not statistically significant for mature milk *(Fig. 1-B)*.

Compositional Analysis of the Milk Microbiota of Different Goats

The matrix of Bray-Curtis distance was calculated based on the OTU abundance of all samples. According to these distance matrices, the similarity analysis of unweighted Unifrac reflected that the difference between these two groups was significant (PERMANOVA, P<0.01) *(Fig. 2)*. The principal coordinates analysis (PCoA) showed that all colostrum and mature milk samples were scattered into two clusters, and there were significant differences between the milk microbial compositions of goats with different feeding styles (PERMANOVA, P<0.01). Specifically, the milk microbiota from indoor-fed goats were mainly grouped in cluster A, while those from pasture-raised goats were mostly aggregated in cluster B *(Fig. 2)*. Of all explained variances, the principal component accounted for 85.7% (PC1) and 5.9% (PC2) for colostrum, and 66.9% (PC1) and 19.7% (PC2) for mature milk.

Top Genera and Taxa Level Analysis of the Milk Microbiota of Different Goats

The relative abundance of the top genera of colostrum and mature milk from different goats were further analyzed *(Fig. 3-A,B)*. Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) was carried out to identify the most differentially abundant taxa with a log LDA value >2.0 and P value <0.05 (Wilcoxon test). The taxonomic differences of colostrum (9; 7 phylotypes) and mature milk (14; 36 phylotypes) from pasture-raised and indoor-fed goats was shown in *Fig. 4*. Specifically, for colostrum, *Propionibacterium* were significantly abundant in pasture-raised goats while *Brachybacterium* were enriched in indoor-fed goats *(Fig. 4-A)*. For mature milk, the relative abundance of *Betaproteobacteria* was higher in pasture-raised goats while *Firmicutes* were enriched in indoor-fed goats *(Fig. 4-B)*.

In addition, the predominant microbial genera of the colostrum of pasture-raised goats were *Weeksellaceae*, *Lactoobacillus*, *Cloacibacterium* and *Yersinia*. In comparison, those for indoor-fed goats were *Dermabacteraceae*, *Bacteria*,

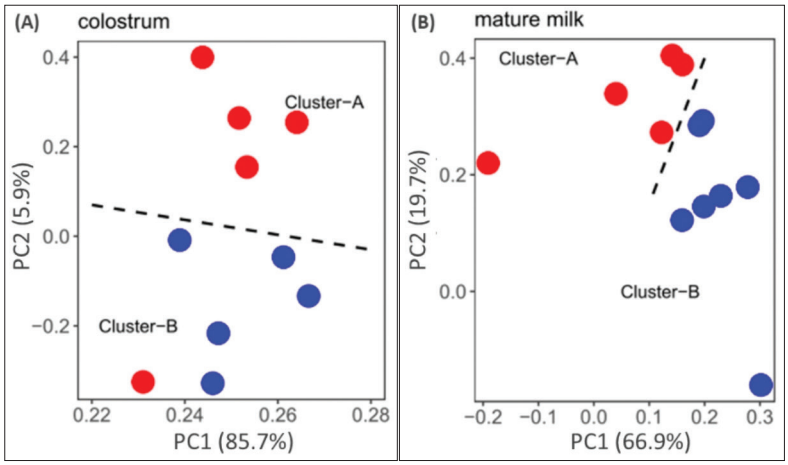

Fig 2. Compositional analysis of the milk microbiota from different farms. PCoA plot of similarities between pasture-raised (P) and indoor-fed goats (I). (A) For colostrum, principal component (PC) 1 and 2 accounted for 85.7 and 5.9% of the variance, respectively. (B) For mature milk, principal component (PC) 1 and 2 accounted for 66.9 and 19.7% of the variance, respectively

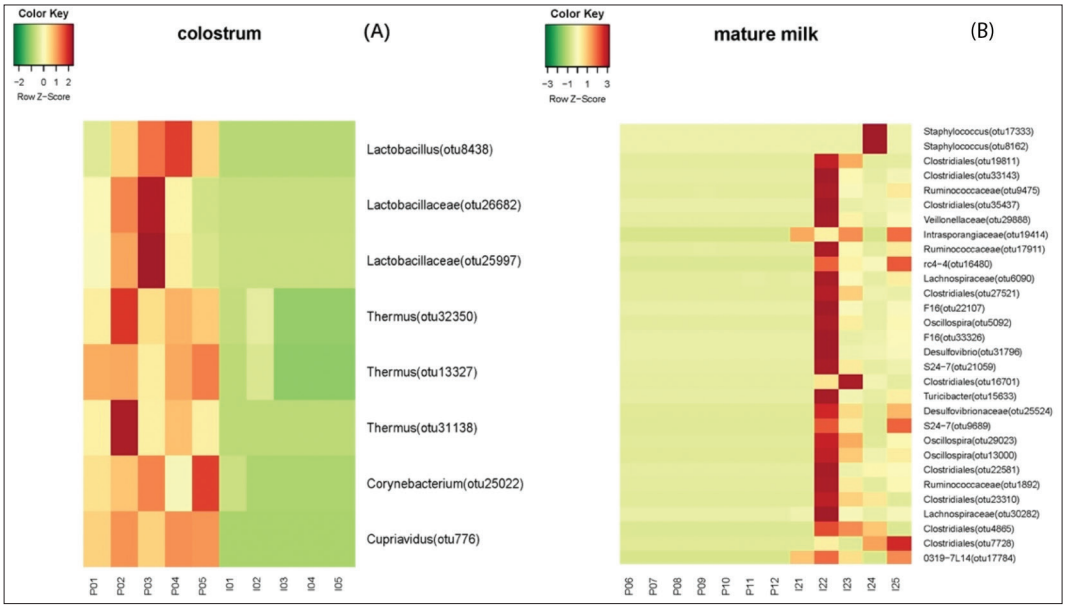

Fig 3. The relative abundance of the top genera of colostrum and mature milk from pasture-raised (P) and indoor-fed (I) goats. The genera with relatively high values are represented in red (0-2) and low values in blue (0-(-2)). Goat milk microbiota composition at genus level was significantly different in goats with different feeding styles (A and B)

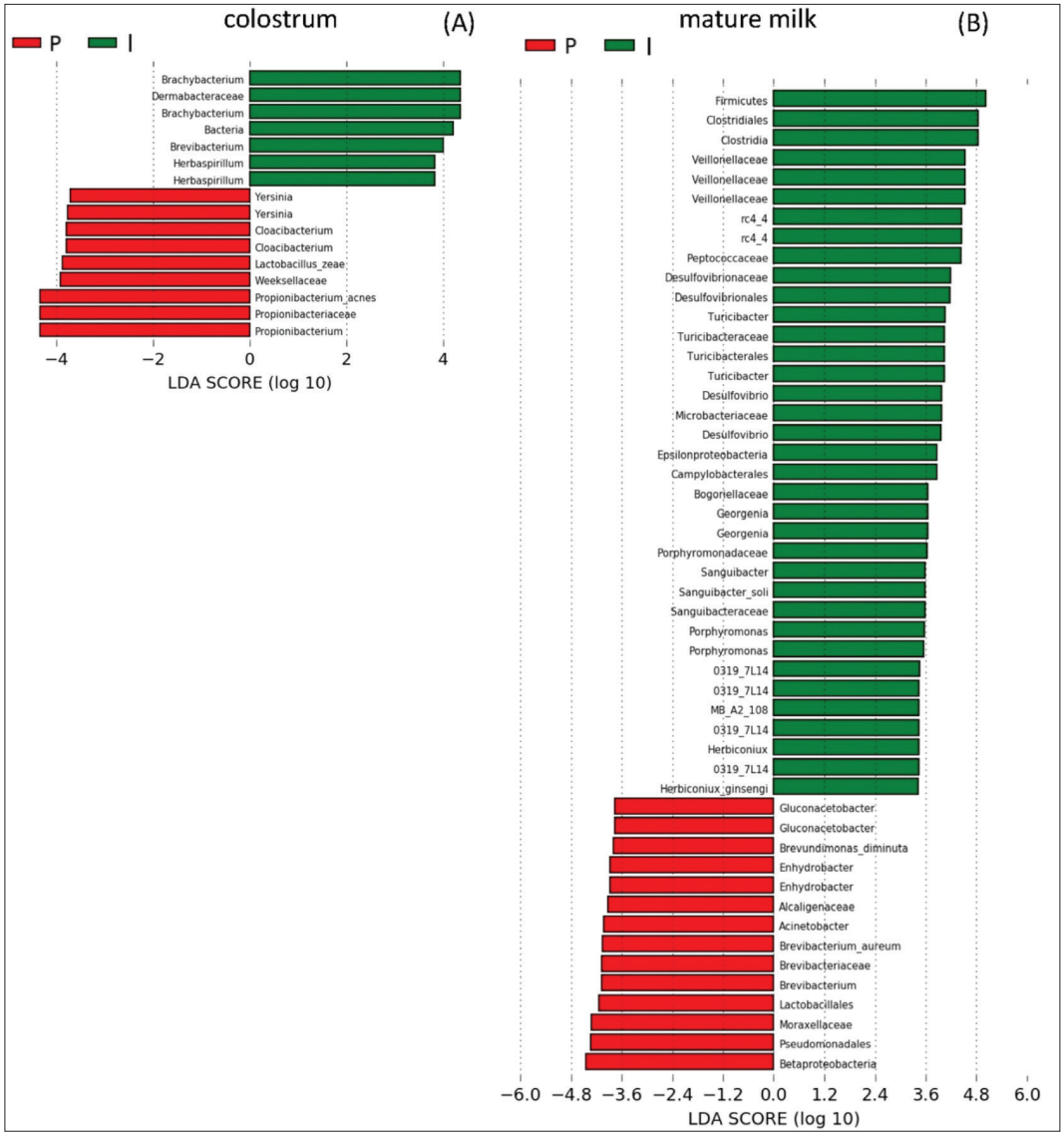

Fig 4. Linear discriminant analysis effect size (LEfSe) analysis of colostrum and mature milk microbial taxonomy. Different taxa levels were measured using linear discriminant analysis (LDA) with effect size algorithm. For colostrum (A) and mature milk (B), histograms of linear discriminant analysis of 16S rDNA sequences were performed with |LDA score| >2 (log10). Pasture-raised goats-enriched taxa were shown with negative LDA values (red), and taxa enriched in indoor fed goats were displayed with positive LDA values (green)

Brevibacterium and *Herbaspirillum (Fig. 4-A)*. The predominant microbial genera of the mature milk of pasture-raised goats were *Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas* and *Gluconacetobacter,* while those for indoor-fed goats were *Clostridiales, Veillonellaceae, Peptococcaceae, Desulfovibrionaceae, Turicibacter, Microbacteriaceae, Campylobacterales, Bogoriellaceae, Georgenia, Pophyromonadaceae, Sanguibacter* and *Herbiconiux (Fig. 4-B)*. The relative abundance of milk microbial taxonomy of these differently-fed goats estimated through LEfSe was significantly different ($P<0.05$).

Functional Characterization of Milk Microbiota from Pasture-Raised and Indoor-Fed Goats

In order to detect the roles of milk microbiota in different farms, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) program was performed to predict the metabolic functions of microbial genes. Base on the Kyoto Encyclopedia of Genes and Genomes pathway (KEGG, *http://www.genome.jp/kegg/pathway.html)* database, the metabolic pathways were sorted into six categories, including genetic information processing, cellular processes, metabolic pathway, metabolism, environmental information processing and organism systems and human diseases.

Significantly differed pathways of colostrum microbiota from the two groups of goats contained transport and catabolism ($P=0.0005$), metabolism of terpenoids and polyketides ($P=0.0006$), signaling molecules and interaction ($P=0.0007$), lipid metabolism ($P=0.001$), transcription ($P=0.001$),

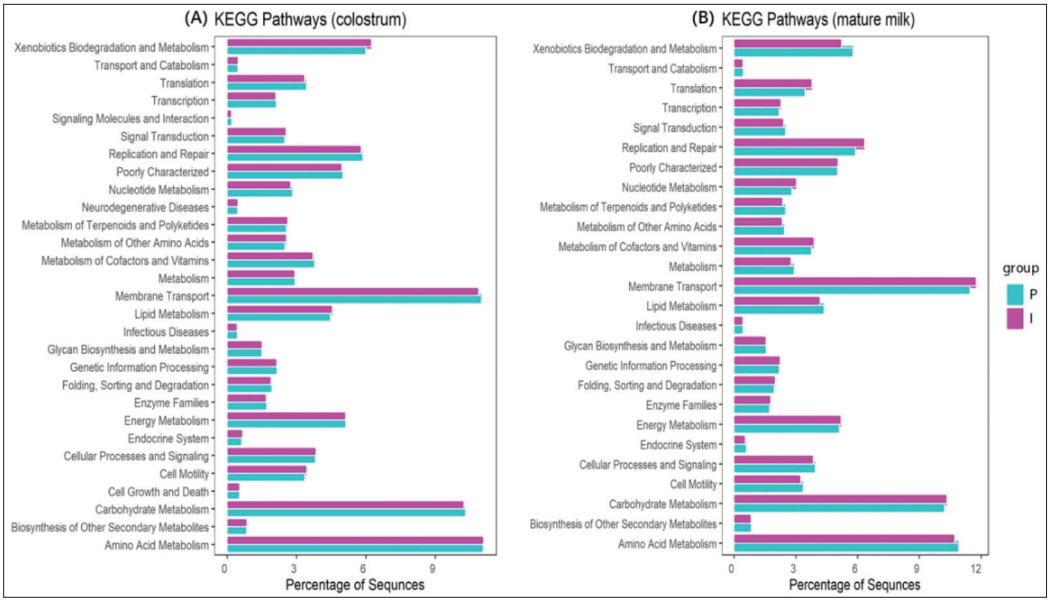

Fig 5. The major categories of the functional analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Relative abundance of microbial genes was compared between pasture-raised goats (P) and indoor-fed goats (I)

metabolism (P=0.002), membrane transport (P=0.002), carbohydrate metabolism (P=0.002), metabolism of other amino acids (P=0.002), infectious diseases (P=0.003), amino acid metabolism (P=0.003), excretory system (P=0.004), biosynthesis of other secondary metabolites (P=0.006), environmental adaptation (P=0.007), enzyme families (P=0.007), nervous system (P=0.008), signal transduction (P=0.008) and energy metabolism (P=0.008) *(Fig. 5-A)*. The significantly differed pathways of mature milk microbiota contained replication and repair (P=0.01), nucleotide metabolism (P=0.01), amino acid metabolism (P=0.005) and membrane transport (P=0.003) *(Fig. 5-B)*.

Discussion

Beyond providing abundant nutrient substances for neonates, mammalian colostrum and mature milk also contain plenty and diverse bacteria that play an important role in modulating the gut microbiota colonization and maturation of the offspring, influencing the health condition of a lactating animal, and affecting the quality of dairy products [8-10,16,17]. Factors from the host and the external environment both critically affect the composition of the milk microbiota [18-20,22,23]. However, it is currently unclear whether pasture and indoor feeding, two major feeding styles of goats, have an effect on milk microbiota. This study analyzed the milk microbiota profile of goats with different feeding styles. The results suggested that pasture and indoor feeding affected milk microbiota composition profoundly and differently, reflected by higher milk microbial diversity and composition in goats from pasturing goats.

Several studies have reported that the animal husbandry practices and diets could influence the rumen and fecal microbiota of goats [26,27]. Consistent with the finding of our study, an earlier study showed that *Proteobacteria* and *Firmicutes* were the major phyla of goat milk microbiota [1]. Interestingly, *Proteobacteria* and *Firmicutes* were also reported to be the predominant phyla in human breast milk [28]. In our research, different feeding styles significantly influenced the major phyla and genera of goat milk microbiota. *Firmicutes* and *Brachybacterium* are the largest phylum of colostrum and mature milk microbiota of indoor-fed goats, while *Propionibacterium* and *Betaproteobacteria* are most enriched in that of pasture-raised goats *(Fig. 4)*. *Firmicutes*, *Proteobacteria* and *Propionibacterium* are also the most abundant phyla of cow milk microbiota, although their specific function is yet to be investigated [29]. Supporting these findings, Zhao et al.[30] indicated that *Proteobacteria* and *Firmicutes* were the predominant phyla in camel milk.

The genus *Lactobacillus* in the colostrum was significantly higher in pasturing goats *(Fig. 3-B)*. This genus contains phylogenetically diverse strains of bacteria and many of the species are commonly used as probiotics. In fact, emerging evidence suggests that supplementation with *Lactobacillus* has multiple benefits, including promoting energy harvesting [31], stimulating the immune system [32], defending against infections [33] and combating fatigue [34]. The reason why *lactobacillus* is enriched in pasture-raised goats is not clear, but this finding provides good evidence that milk from pasture-raised goats may be more beneficial from a probiotics point of view.

PCoA clustering analysis showed that the bacterial structure of goat milk is different between goats with different

feeding styles *(Fig. 2)*. This result displayed that all samples were clustered in two different groups, suggesting that the milk microbial community of goats of the same farm were highly conserved. This phenomenon may be caused by the fact that these goats have adapted to their living styles, including different diets and living environment. Besides, several other factors could also impact the milk microbial community, i.e., genetic specificity, geographic location, lactation stage, feeding and milk transportation and storage [35]. Thus, the microbial community of the milk is the comprehensive activity of all the influencing factors. However, the present study could not rule out influencing factors other than feeding styles.

In conclusion, this study analyzed the microbiota of colostrum and mature milk of pasture-raised and indoor-fed goats and showed that these feeding styles could profoundly influence the abundance and diversity of milk microbiota.

Ethical Approval

Not applicable.

Acknowledgements

The authors would like to thank Mrs. Li Wang of NWAFU for her technical assistance.

Financial Support

This research was supported by National Natural Science Foundation of China (31902282), Basic Research Plan of Natural Science of Shaanxi Province (2020JQ-270), Key Projects of Science and Technology Coordination in Shaanxi Province (2020ZDLNY02-06) and Qinghai province Major R&D and Transformation Project (2021-QY-211, 2018-NK-125).

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

W.M. and D.C. proposed and supervised this study. T.J. (Tian Jing), X.Y., H.F. and F.C. analyzed the data. T.J. (Tiantian Ji), J.D. and X.Y. wrote the first version of the manuscript. H.Z., T.W. and X.C. edited the manuscript. All authors have reviewed the final version of this article. All authors have read and agreed to the published version of the manuscript.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 649-653, 2021
DOI: 10.9775/kvfd.2021.25734

SHORT COMMUNICATION

The Productivity Evaluation of Madura Cattle Under Beef Cattle Research Station Breeding Management

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Article ID: KVFD-2021-25734 Received: 11.03.2021 Accepted: 08.07.2021 Published Online: 17.07.2021

Abstract

Madura cattle is one of the local beef cattle that have high adaptability to the influence of tropical environments and is one of the local germplasms that must be maintained and increased in productivity. This study aimed to evaluate the productivity of Madura cattle under Beef Cattle Research Station (BCRS) Breeding Management. The Madura cattle were 138 dams and 6 sires, which come from the selective collection. They were developed under BCRS breeding management, such as, application of natural mating in Litbangtan communal-pen models with the sex ratio of 1 male for 15-20 females. The parameters were first mating age, first partus age, calving interval (CI), days open (DO), calving rate (CvR) and calf crop (CC). The results showed that the first mating age of Madura cattle was (average ± SE) 23.2±1.26 months, first partus age 32.7±1.25 months. Madura cattle had the shortest CI 358.1±22.1 days with DO 78.1±22.1 days, CvR and CC 70% and 90%, respectively. The age of first mating and partus of Madura cattle come from the selective collection were similar with Madura cattle in their original place. It was because of the identical mating pattern; while the CI and DO value got better with time of observation. It was concluded that developing Madura cattle under BCRS breeding management improved their reproduction value.

Keywords: *Beef Cattle Research Station, Breeding management, Madura cattle, Productivity*

Besi Sığırı Araştırma İstasyonu Islah Yönetimi Kapsamında Madura Sığırlarının Verimliliğinin Değerlendirilmesi

Öz

Madura sığırı, tropikal ortamların etkisine yüksek adaptasyon kabiliyetine sahip yerel besi sığırlarından birisi olup, korunması ve verimliliğin artırılması gereken yerel gen kaynaklarından biridir. Bu çalışmada, Besi Sığırı Araştırma İstasyonu (BCRS) Islah Yönetimi kapsamında Madura sığırlarının verimliliğinin değerlendirilmesi amaçlamıştır. Selektif koleksiyondan138 dişi ve 6 erkek Madura sığırı seçildi. Bunlar, Litbangtan ortak kafes modeli ile 15-20 dişi için 1 erkek olacak şekilde doğal çiftleşme yöntemi ile BCRS Islah Yönetimi kapsamında büyütüldüler. Parametreler, ilk çiftleşme yaşı, ilk doğum yaşı, buzağılama aralığı (CI), servis periyodu (DO), buzağılama oranı (CvR) ve buzağı verimi (CC) idi. Sonuçlar, Madura sığırlarının ilk çiftleşme yaşının 23.2±1.26 ay ve ilk doğum yaşının 32.7±1.25 ay (ortalama ± SE) olduğunu göstermiştir. Madura sığırları, 78.1±22.1 günlük servis periyodu ile en kısa buzağılama aralığına (358.1±22.1) ve %70 buzağılama oranı ve %90 buzağı verimine sahipti. Selektif koleksiyondan gelen Madura sığırlarının ilk çiftleşme yaşı ve ilk doğum yaşı, orijinal yerlerindeki Madura sığırlarına benzerdi. Bunun nedeni, aynı çiftleşme modelinin uygulanmasıydı ve buzağılama aralığı ve servis periyodu değerleri gözlem süresince daha iyi hale geldi. Madura sığırlarının BCRS Islah Yönetimi altında yetiştirilmesinin üreme değerlerini iyileştirdiği sonucuna varılmıştır.

Anahtar sözcükler: *Besi Sığırı Araştırma İstasyonu, Islah yönetimi, Madura sığırı, Verimlilik*

INTRODUCTION

Madura cattle is one of the Indonesian local beef cattle that still exists and widely developed in East Java, especially Madura Island. As an asset of genetic resources (germplasm) local to Indonesian cattle, the existence of Madura cattle must be maintained both in terms of population and genetic purity [1].

How to cite this article?

Hartati H, Luthfi M, Khrisna NH, Sukmasari PK, Fitrayady HP, Widiyawati R, Dikman DM: The productivity evaluation of Madura cattle under beef cattle research station breeding management. *Kafkas Univ Vet Fak Derg,* 27 (5): 649-653, 2021.
DOI: 10.9775/kvfd.2021.25734

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Madura cattle have some advantages such as they have good growth in poor quality of forage, have high adaptability to tropical environments, and can run fast, hence it is usually used for racing (karapan), and has a good body appearance, so it is also used as an "exhibition" cattle (sonok). Madura cattle also has a percentage of carcasses is high with good-quality meat [2]. These advantages can be a good reason to put them as local cattle for meat producers like other local beef cattle.

The beef cattle population in Indonesia was approximately 17.118 million in 2019, however, the real population of madura cattle has not yet been confirmed. DGLS [3] reported Madura cattle population was approximately 635.000 or 5.16% of the total beef cattle population in 2010 (12.3 million). They are spread out in four districts in Madura Island. So far, the breeding activities of these cattle have focused more on producing superior cattle through coincidental selection. Especially in Madura Island, as the Madura cattle development area, selection serves more to produce Karapan cattle, namely bulls that are able to run fast, agile and have muscular skeletons [4] and Sonok cattle are tame and beautiful-looking cows [5] and is usually selected based on the superior female pedigree [6]. Karapan and Sonok cattle are the diversity of traditions and cultures as well as a portrait of the community's love for Madura cattle. Nurlaila and Zali [7] also reported that Madura cattle not according to these criteria will be used as regular beef cattle. This is the reason why Madura cattle still exist and thrive in Madura Island.

But lately, the reproductive performance of Madura cattle has reportedly begun to decline markedly by high calf mortality, long calving intervals, low birth rates and slow genetic improvement. Calving Interval (CI) and Days Open (DO) are two common measures used to determine the reproductive performance of livestock. The mean length of the calving intervals of a cow determines to the large extent her total productivity [8]. Karnaen et al.[9] also reported that the range of Madura cattle birth weights in Madura Island was just from 16 to 17 kg while the range of weaning weights was from 76 to 80 kg.

Improvement of Madura cattle productivity through breeding activities is still being done both through genetic improvement and selection. Basically, animal productivity is influenced by genetic (internal) and environmental (external) factors as well as the interaction of these two factors [10]. Therefore, the success of the productivity improvement of Madura cattle can only be achieved if genetic improvements are followed by improving environmental conditions, so the selected cattle have the opportunity to fully express their genetic potential.

The Indonesian Beef Cattle Research Station *(Loka Penelitian Sapi Potong Grati)* as one of the Technical Implementation Unit (TIU) under the Indonesian Agency for Agricultural Research and Development, Indonesian Ministry of Agriculture has developed Madura cattle from 2013 until now. The Madura cattle breeding pattern uses a BCRS breeding management that keeps the cattle in a Litbangtan communal pen. One of the strengths of this management system is efficiency in effort and time that have some good impact. The main good impact was able to shorten the calving interval; therefore, it directly improves reproductive efficiency. This study aimed to evaluate the productivity of Madura cattle that were developed under BCRS breeding management.

Materials and Methods

Ethical Approval

The following experiment was conducted under the guidelines of the Indonesian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Indonesian Ministry of Agriculture Animal Ethics Committee (Balitbangtan/Lolitsapi/Rm/14/2019).

Animal and Data Collection

Data were collected from the herd of Madura cattle in BCRS at Grati, Pasuruan, East Java for 6 years from 2014 to 2019. Productivity data were from 138 Madura dams and 6 Madura sires with the age range of 1-year-old. The variables observed were the body weight of dams and sires, gestation detection, date of birth, calf sex, and birth weight.

Breeding and Feed Management

The research method was carried out with recorded variable data periodically as requirements on a certain physiological status. As a breeding concept, the BCRS breeding management referred to the concept of good breeding management that considers the environmental impact as well as the genetic. This concept was used as a treatment in this research.

The Litbangtan communal pen was used (3 m^2/head) for the main pen in the current research. This pen was used as a mating and gestation pen until 8 months of gestation. Individual pens were used for prospective sires and sires which were not being in use for mating; while lactation pens were used for dams that were gestation by 8 months, give birth, and continued by lactating up to 40 days of calf age. The mating system applied was referred BCRS breeding model also. The model was carried out by the 15 to 20 steady heifers/dams placed in a communal pen together with a selected sire. Furthermore, the first gestation detection (rectal palpation/RP) was conducted 3 months after mating and the second one was done in the following month or 4 months after mating.

Standard feeds were used in this experiment, and the feeds consist of common roughage (elephant grass and rice straw) and additional feed which was mixed from the

agricultural, plantation, and agroindustries byproducts. The nutrient contents were about 8-9% crude protein (CP), 57-60% total digestible nutrient (TDN), and 20-22% crude fiber (CF). The ration ingredients and its formulation were not permanent, it depends on the availability of feedstuff. The ration was adjusted to the physiological status of the cattle. Generally, cattle consumed 2.5-3.5% of their body weight.

Data Analysis

Observations on reproductive performance were recorded periodically; the variables were gestation cattle, birth rate, date of birth, body weight at birth. The other supporting variables were also recorded such as body weight and body size. All variable collected data were analyzed and used to determine some parameters descriptively. The parameters were the first mating age, first partus age, calving interval, days open, calving rate and calf crop.

The first mating age was calculated by subtracting the birth date from the estimated gestational age for 280 or 285 days. The calving interval (CI) was the amount of time (days or months) between two birth times on the same cow sequentially. Days open (DO) was calculated as the time period between calving and conception in a given cow [11]. Calving rate (CvR) is the percentage of the number of birth calves as a result of the first insemination or subsequent insemination.

Results

Study on the productivity evaluation in Madura cattle showed that the average body weight and the shoulder height of Madura dams were 178.9±2.5 and 110.2±0.04 while Madura sires were 236±6.0 and 130.1±0.08, respectively. The performance of Madura cattle resulted from the selective collection is presented in *(Table 1)*. In this study, the reproductive performance of Madura dam specifically first mating and first partus age on BCRS breeding management *(Table 2)*. Calving interval data are almost certainly obtained

***Table 1**. The average body weight and shoulder height of madura dams and sires*

Sex	n	BW+SEM	SH+SEM
Dams	123	178.9±2.5	110.2±0.04
Sires	6	236.0±6.0	130.1±0.08

***n:** number of samples; **BW:** body weight; **SH:** shoulder height; **SEM:** standart error of mean*

***Table 2.** First mating and partus of Madura dams*

Reproduction Variable	n	Month+SEM
first mating age	124	23.2±1.26
first partus age	124	32.7±1.25

***n:** number of samples, **SEM:** standar error of mean*

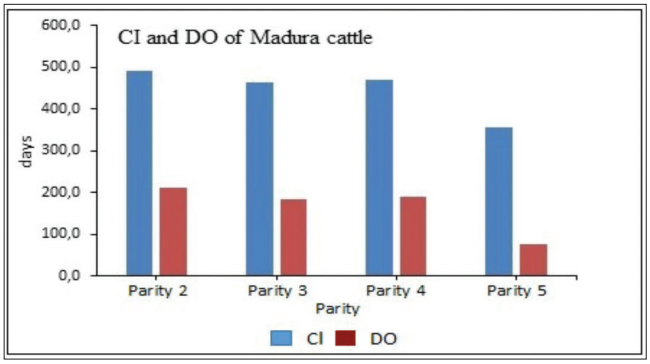

Fig 1. Calving interval and days open of Madura cattle

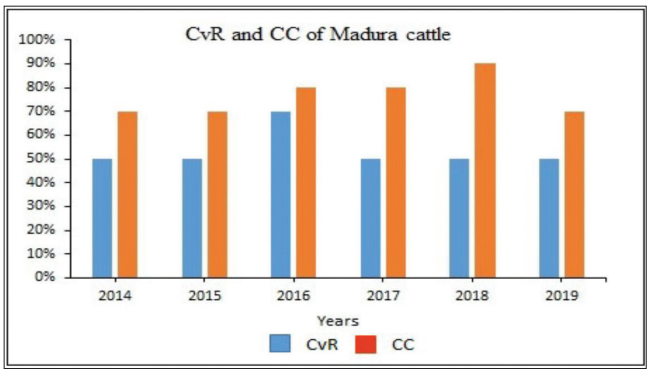

Fig 2. Calving rate and calf crop of Madura cattle

from the second partus point in different years, so the data would be easier to interpret if presented based on the parities, as shown in *Fig. 1*. CI value *(Fig. 1)* in the second parities was 491.4±14.6 days, while the third and fourth parities were almost the same and were 462.8±18.6 days and 469.3±25.1 days and the shortest CI value obtaining at fifth parities was 358.1±22.1 days. The variety of CI values in BCRS was largely occurred by the length of the DO. The DO values of Madura cattle in this study were 211.4±14.6, 182.8±18.6, 189.3±25.1 and 78.1±22.1 days for consecutive observations based on parities. In addition, productivity performance also included Calving Rate (CvR) and Calf Crop (CC) and shown in *(Fig. 2)*. The calving rate of Madura cattle was quite high and varied from year to year between 50% until 70%. In BCRS, Madura calf crop in the last six years recorded (2014 to 2019) fluctuated from 70 to 90% (average 74%).

Discussion

The body weight and shoulder height of Madura dams and sires come in quite high. Both of them were higher than the standards set by the Ministry of Agriculture no. 3735/Kpts/HK.040/11/2010. The standard stated shoulder height of Madura sire at 24-36 months was 105 cm and for Madura dam was about 102 cm [12]. While the Indonesian National-Standard, SNI 7651.2: 2013 issued by National Standardization Agencies (BSN) reported the shoulder

height on the first class of quantitative requirements at 12-<18 months was 122 cm for Madura sire and 116 cm for Madura dam [13]. The mean shoulder height of Madura sire in this study (130.1±0.08 cm) is higher than in the Class I category in Indonesian National Standard, which is 12-<18 months and 122 cm for Madura sire and while the mean shoulder height of the Madura dam (110.2±0.04 cm) is lower from the size of the Indonesian National Standard and is 116 cm [13]. The result was in agreement with Nurgiartiningsih et al.[14] reported cow body weight was one indicator for livestock productivity, which can be predicted by the size of the linear body of the cow. The high correlation indicated that chest girth could be used as a parameter for estimating body weight of female Madura cattle.

The animal genetic potential was measured based on their production and reproductive performances. Quantitative data of biological-potential on production and reproduction performance phenotypes were not be separated from the environmental influence where the livestock kept. The result in this study showed that age was similar to the Madura-cattle first mating age developed in Pamekasan Regency, Madura Island, which was reported 23.44±2.57 months [15]. Kutsiyah [2] reported the age was relatively lower in Sapudi Island (21.12±0.16 months) and in Sumenep and Pamekasan Regency were 19.85±0.81 [16].

Early pregnancy is the key to successful livestock production [8]. The first mating age of an animal is very closely related to the management of both feeding and mating management. The Madura cattle were directly placed together in the Litbangtan communal pen since their coming in 2013. A communal pen was entered by 15-20 sire with a dam, the pen was also used as a mating pen. This model leads to the first mating age faster; the Madura cattle first mating age in BCRS occurred before two years. The first mating age of Madura cattle in BCRS was an estimation because the animal mating system was natural and nobody knew precisely the mating time. It can be counted down from the first partus age, the length of the gestation period on Madura sire is 280-285 days.

The first partus age of Madura cattle under BCRS breeding management was relatively same as the results of Nurlaila et al.[17] and Wisono et al.[15] study in Pamekasan, Madura, that reported 33.92±3.88 months and 34.63±2.46 months, consecutively. Meanwhile, Kutsiyah [2] reported the first partus age of Madura cattle in Sapudi Island was rather better 31.97±6.43 months.

The reproduction status of Sonok cattle in Pamekasan District was quite good. The first age of mating was 23.40±4.17 months, it was relatively the same as Madura cattle under BCRS breeding management 23.2±1.26 months. By these observations both on first mating and partus age indicated the BCRS breeding management took a good influence on the reproductive performance of Madura cattle. Many factors affect the reproductive performance of beef cattle including the type of feed consumed, feed quality, and peripartum diseases.

Calving interval (CI) is the time between two successive calvings so that it is only available for cows from the second parity onwards, in tropical and subtropical regions between 12-14 months. Calving interval can be divided into three periods, gestation, post-partum anoestrus (from calving to first estrus) and service period (first postpartum estrum to conception) [18]. The ideal calving interval is 12 months, which is 9 months for the gestation and 3 months for days open (DO) which defines the number of days between calving and the next conception. The days open may be used to provide exclusive lactation for the calves. However, calving interval are also affected by the production system, the length of postpartum estrus, length of gestation, and length of gestation is influenced by genetic and environmental factors [19]. A good reproductive performance is when a dam able to produce one calf in one year.

The CI values in this study tended to vary based on parities and it was different of CI of Madura cattle on Sapudi Island was 442.9±65.70 days [3]. The variety of CI values in BCRS was largely occurred by the length of gestation and the length of the DO. A longer DO result in a higher CI value and this circumstance is a debilitate for breeding activities. The DO value in this study was different from the report of Nurlaila et al.[17] amounting to 168.67 days. The difference in CI and DO values in Madura cattle breeding was most likely caused by differences in feed and variations in feed ingredients, breeding patterns, and mating management. CI is mainly determined by the open day between birth and conception and influenced by the cycles of estrus, reproduction, and conception. Breeding management which also affects the CI value includes feeding, time of insemination or time of heat detection.

The highest CvR was obtained in 2016 of 70%, the value indicates that Madura cattle have a good reproductive efficiency reflected in a high CvR, the value was about 55-65%. The results of other studies show that of the 77 cattle that were mated, 56 cattle were conception or pregnant at the first mating with Conception Rate (CR) was 73%. Calving rate was the most important trait, indicating that the economic effect of the reproductive performance is very high in an integrated beef production system. The size of this CR was also due to quality good stud used in mating, improvement efforts management in heat detection by observing the behavior of cows, namely, indicates a reduced appetite, restless behavior, and often comes out mucus, swollen, red, wet, as well the accuracy of the mating performed. The calving rate can reach 62% on two times perfect insemination, without producing a calf, artificial insemination considered to be unsuccessful. Several factors determine the low CvR value were embryo

death and feeding of cows not in accordance with their conditions and physiological.

Calf crop is the number of calves weaned to a particular cow group in percent. In this study, the average value of Calf Crop was 74% almost the same as the studied by Susilawati [20] at about 75%. Increasing the calf crop can be done by paying attention to the time and length of estrous, the accuracy of mating, nutrition and disease control. Based on the performance progress of Madura cattle outside Madura Island (BCRS), it has shown several increased reproductive performances. Therefore, the condition of Madura cattle can be used as the basic population for performance testing and breeding programs to achieve an increase in the genetic quality of Madura cattle to support the conservation program for Madura cattle as local genetics Indonesian resources.

In conclusion, the performance of Madura cattle under Beef Cattle Research Station breeding management showed improvement on their productivity value including the values of CI, DO, CvR and CC.

Acknowledgement

This study was funded by The Indonesian Agency for Agricultural Research and Development (IAARD). The author would like to thank all the Indonesia Beef Cattle Research Station of Grati Pasuruan teams who helped carry out this research.

Conflict of Interest

Authors declares that there is no conflict of interests.

Author Contributions

HH planned, designed the research, analyzed all data and drafted manuscript. ML, NHK, PKS, HPF, RW and DMD contributed and help in the research. All authors concurred with the submission and subsequent revisions submitted by the corresponding author.

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Kafkas Universitesi Veterinar Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 655-659, 2021
DOI: 10.9775/kvfd.2021.25927

CASE REPORT

Optimal Management to Improve Quality of Life for an Injured Baby Elephant: Thailand Multidisciplinary Care Team

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Article ID: KVFD-2021-25927 Received: 22.04.2021 Accepted: 28.07.2021 Published Online: 01.08.2021

Abstract

This article aimed to report the care, management, and monitoring of an injured female baby elephant. The collaboration among the multidisciplinary team included veterinary medicines, elephant handling, engineering, and physical therapy in the 3-year-old elephant with its left forelimb phalanges amputated through being caught in a snare trap. The management comprised medical wound care, nutrition and applying a prosthetic shoe. The kinematic parameters and vital sign monitoring were analysed. The results show that biomechanics gait analysis and physiological responses revealed promising benefit of the prosthetic shoe by reflecting a greater symmetrical gait pattern without dyspnea and no sign of exertion during daily life activity.

Keywords: *Amputation, Artificial shoe, Elephant, Gait analysis, Prosthesis*

Yaralı Yavru Bir Filin Yaşam Kalitesini Artırmak İçin Optimal Yönetim: Tayland Multidisipliner Bakım Ekibi

Öz

Bu makalede, yaralı bir dişi yavru filin bakım, yönetim ve izlenmesinin bildirilmesi amaçlanmıştır. Multidisipliner ekip arasındaki işbirliği, kapana yakalanarak sol ön ayak falanksları kesilen 3 yaşındaki bir fil için veteriner ilaçları, filin bakımı, mühendislik ve fizik tedaviyi içeriyordu. Yönetim, tıbbi yara bakımı, beslenme ve protez ayakkabı uygulanması şeklindeydi. Kinematik parametreler ve vital bulgu takibi analizleri gerçekleştirildi. Sonuçlar, biyomekanik yürüyüş analizi ve fizyolojik tepkilerin, dispne ve günlük yaşam aktivitesi sırasında hiçbir efor belirtisi olmaksızın daha simetrik bir yürüyüş modelini yansıtarak protez ayakkabının umut verici faydasını ortaya koyduğunu göstermektedir.

Anahtar sözcükler: *Amputasyon, Fil, Protez, Yapay ayakkabı, Yürüyüş analizi*

INTRODUCTION

In Thailand, elephant poaching is illegal, and in most cases a "snare trap" is used. This often results in an injured baby being left behind with lack of its mother's milk. It would then develop severe ailments, for instance, mental health and major musculoskeletal problems and sometimes die from infectious diseases such as elephant endotheliotropic herpesvirus (EEHV) [1]. This report described collaborative health care management, introduced a guideline for elephants with amputated forelimb phalanges and monitored the consequences on walking characteristics after wearing a prosthetic shoe, by using motion analysis to evaluate the elephant's quality of life (QOL).

CASE HISTORY

A 3-month-old wild-born female elephant was referred from a private tourist camp to the National Elephant Institute (NEI). She was trapped at the left carpal joint by a

How to cite this article?

Kongsawasdi S, Wantanajittikul K, Langkaphin W, Chuatrakoon B, Namwongprom K, Prupetkaew P, Angkawanish T: Optimal management to improve quality of life for an injured baby elephant: Thailand multidisciplinary care team. *Kafkas Univ Vet Fak Derg,* 27 (5): 655-659, 2021. DOI: 10.9775/kvfd.2021.25927

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snare when she was 1 year old, and developed a clinical fever and fetid smell. Trans-metacarpal, partial foot amputation was performed as primary treatment by the veterinarian at the tourist camp, and antibiotics, skin flap and wound management were administered. She was then transferred to the NEI for appropriate management.

Health Care and Management

She was fed mainly with grass (protein of around 4-5%) and fruit supplements, while staying in a barn (4x4 m with sand bedding) at night and spending daytimes in natural forest. Minerals and vitamin C were provided daily and walking exercise for 1 h twice per day. Contagious diseases such as EEHV were monitored monthly.

Fig 1. Development of abnormal alignment of the right forelimb before the shoe was applied

Development of a Prosthetic Shoe

Initially, the length of discrepancy between right and left forelimbs was 4 cm (right or intact side was 139 cm, and left or amputated limb 135 cm). As she was unable to bear weight on her left forelimb, non-used atrophy of the left muscles occurred. Malalignment of the right shoulder and elbow joint also was observed in the frontal plane *(Fig. 1)*, which was caused by the need to shift more weight onto the right side. Her head was at a lower level when compared to the vertebrae and pelvis. Consequently, from the imbalance of weight distribution between the forelimbs and hindlimbs, a sideways twist of the thoracic and lumbar spine developed, forming an S-shaped vertebra *(Fig. 2)*. These deformities might progress further and affect her long-term QOL. Therefore, a prosthetic shoe was used to replace the missing parts of her leg and regain functionality. As this was probably a rare case of a young elephant with limb amputation in Thailand, this project established a multidisciplinary team that worked together to develop an appropriate prototype model. Integral components of prosthetic prescription included a socket, interface and joint unit [2]. Regarding the elephant, the materials used for the socket were important, with concern about adequate mechanical coupling between the soft tissues and supporting device. The materials should be able to manage the heavy load and provide adequate stability, but not excessively, to the point of risking tissue breakdown. Therefore, a prototype shoe was designed by the mechanical engineer team at the Faculty of Engineering, Chiang Mai University in collaboration with the Rubber Authority of Thailand. The prototype shoe was developed from latex foam, using 2 layers of fabric for the coating liners. The insole (shoe floor) density was 508 kg/m^3, rubber hardness unit of 73 Shore OO and side density of 250-260 kg/m^3 with hardness of 41 Shore OO, which enabled the qualification mentioned *(Fig. 3)*.

Walking with the Prosthetic Shoe and Vital Sign Monitoring

The elephant was given the prosthetic shoe for the time

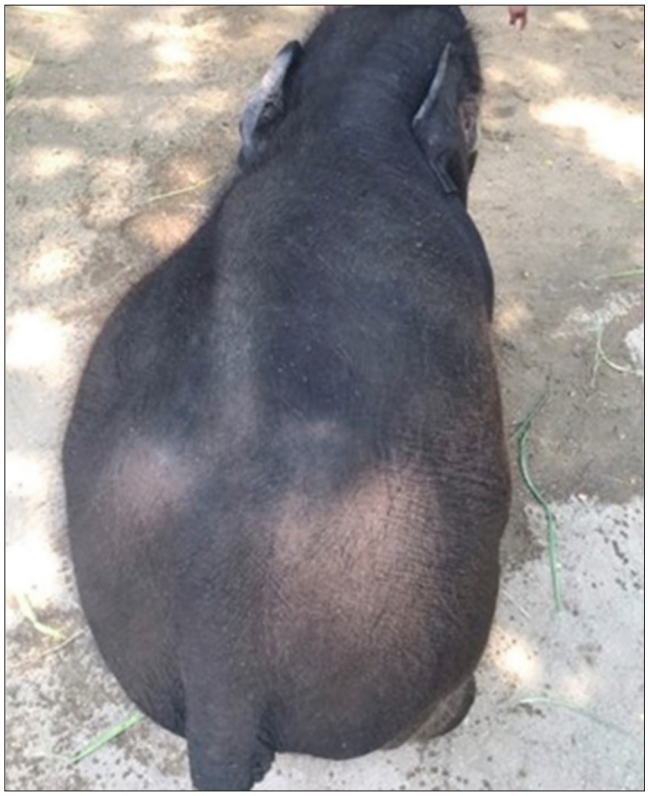

Fig 2. S-shaped scoliosis as a result of uneven weight distribution

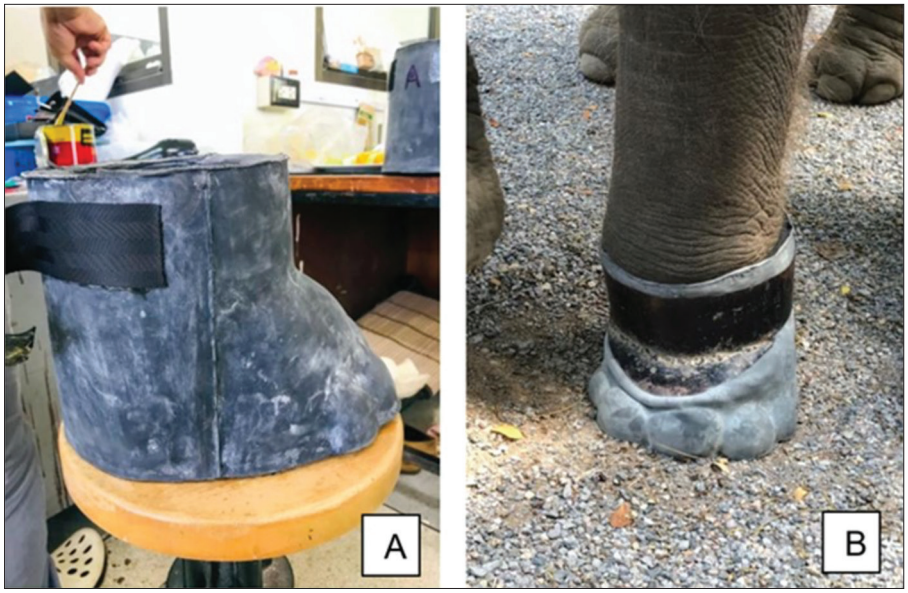

Fig 3. Prosthetic shoe prototype made from latex foam

Table 1. *Vital sign monitoring during daytime with the prosthetic shoe*

Parameters	Normal Range	Minute				
		0	15	30	45	60
Body temperature (°C)	36-37 [4]	36.4	N/A	N/A	36.8	N/A
Respiratory rate (breath/min)	4-12 [5]	8	10	8	8	8
O_2 Saturation (%)	95-100	97	98	92	94	98
Pulse rate (beat/min)	25 -30 [6]	50	54	57	61	61
Systolic blood pressure/Diastolic blood pressure (mmHg)	178.6±2.94/118.7±3.10 [7]	133/82	127/93	129/78	130/88	127/88
Mean blood pressure (mmHg)	144.6±2.9 [7]	95	103	90	101	98

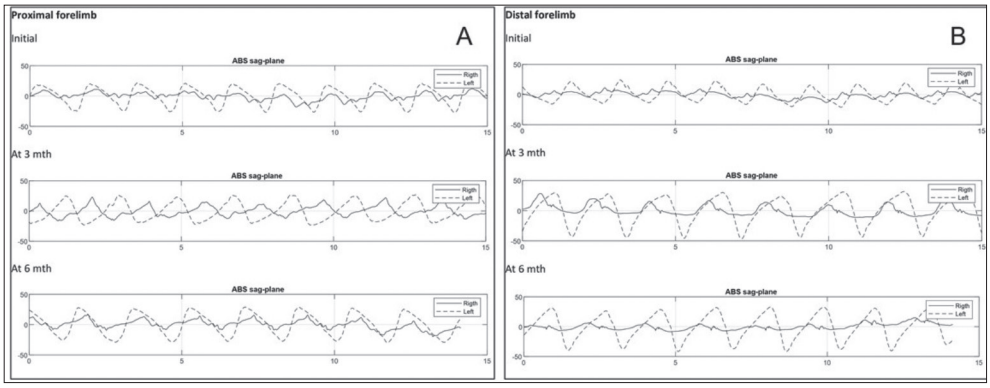

Fig 4. Graphical illustration of proximal (A) and distal (B) forelimb motion

she spent in the forest, which was 3-4 h per day, and the veterinarian team monitored changes of vital signs over 60 min of walking with it *(Table 1)*. All the vital sign parameters, except for high pulse rate, were kept at the normal range, indicating that the elephant had no dyspnea and could maintain pulmonary gas exchange well. However, the heart rate in a young elephant is higher than that in an adult and this elephant could maintain a comparable heart rate from the start to finish of the 60-min walk. Therefore, it could be claimed that her overall physiological responses verified that wearing a prosthetic shoe for 60 min was no burden on her daily living.

Biomechanics Gait Analysis

To quantify walking mobility while wearing the shoe, the biomechanics parameters of gait were analyzed using novel technology of three-dimensional (3D) inertial measurement units with wifi sensors [3]. Data were obtained after applying the shoe for three and six months. The elephant was guided to walk normally at a comfortable speed.

Graphical data demonstrated changes of absolute rotation angle over time, which enabled the movement of each joint segment of the forelimbs and hindlimbs to be

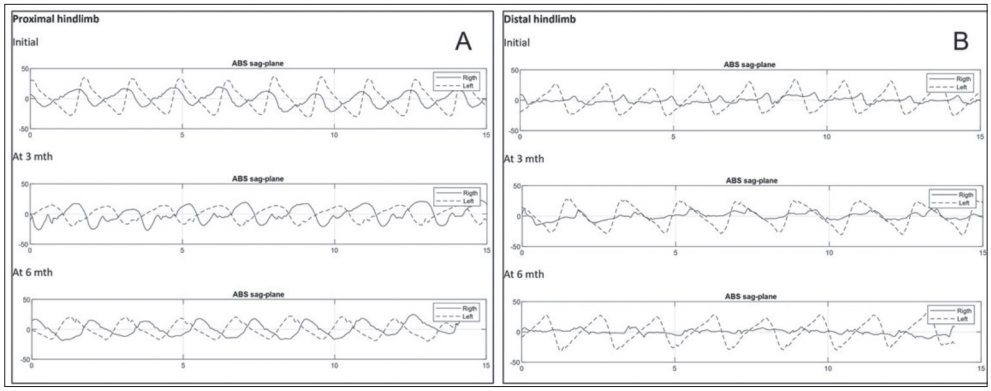

Fig 5. Graphical illustration of the proximal (A) and distal (B) hindlimb motion

Fig 6. The level of head and pelvis after applying the shoe for 6 months

monitored. *Fig. 4-A,B* demonstrates the forelimb motion, and illustrates that in the initial period, both the proximal and distal part of the left forelimb moved to a greater extent than the right one, showing a nonreciprocal pattern (shown in initial phase, *Fig. 4-A,B)*. After 3 months, the proximal part seemed to move more symmetrically and synchronize with the right side. Nevertheless, after 6 months a synchronized pattern between left and right forelimbs occurred, and the left proximal part showed a wider range of movement than the right one.

Analysis of the hindlimb movement *(Fig. 5-A,B)* indicated that both proximal and distal parts of the right hindlimb moved in a very similar manner to the forelimb in the initial period. However, at 3 months, the proximal hindlimb moved more symmetrically and the magnitude of both sides was comparable. After 6 months, the magnitude of the left and right proximal hindlimbs was similar, but not reciprocal, whereas the distal part moved very closely to the forelimb in all movements of the gait cycle. For six-month period of wearing the artificial shoe, it showed promising potential benefits on structural changes. The S-shape twist of the back was to a lesser degree of scoliosis and the head was aligned more symmetry with the pelvis level *(Fig. 6)*.

Discussion

Earlier evidence [8-10] and the authors' previous study [11] revealed that elephants use a lateral sequence footfall pattern and an inverted pendulum mechanism while walking in order to modulate the center of mass and conserve muscular activity. Even though, in this case, the gait cycle of the elephant was nonreciprocal in the initial period *(Fig. 4-A,B)*, it should transit rhythmically between the two phases of footfall pattern. It was postulated that the elephant did not feel comfortable with the artificial limb, and thus moved the right forelimb faster, in order to distribute weight from the left side during the mid-stance period. The right proximal and distal parts of the hindlimb were found to move in a similar manner to the forelimb during the initial period. This might be a consequence of the coordination pattern of the interlimb, from which the elephant tries to conserve the lateral sequence footfall pattern of gait, providing the greatest stability and energy saving. In the three-month period, the proximal hindlimb movement pattern became better, as she familiarized herself with the prosthetic shoe. The proximal part moved more symmetrically and the magnitude of both sides was comparable. Nevertheless, after 6 months, asynchronized pattern between left and right forelimbs occurred again. The left proximal part showed a greater range of movement than the right one, whereas the distal part of the right side demonstrated less movement than the left one. The elephant was trying to keep the right limb rigid and comparable to the left one. Gate deviation could be due to a weathered shoe and increased body weight. Vital signs monitoring when walking with the shoe for at least 60 consecutive minutes indicated normal physiological responses, with no signs of exertion. During six months of wearing the shoe, no observable pressure ulcers on the soft tissue pad or uncomfortable behavior occurred. These findings demonstrated the quality of the shoe, which can restore basic lower-limb functions, i.e., stable support, energy storage and propulsion, and shock absorbance.

In conclusion, although the elephant is an important animal of Thailand, some of them suffer from injuries through many causes such as road accidents, and special care is needed afterwards. This report revealed the health and

QOL of an injured female baby elephant when a prosthetic shoe was applied. The ultimate goal of the prosthesis was to restore the elephant's walking capabilities and support its body weight in a comfortable manner as well as distribute mechanical stresses properly throughout the prosthetic and residual limb. Biomechanics analysis of gait using novel 3D motion analysis revealed promising benefits from wearing the shoe, with an asymmetrical to a more symmetrical gait pattern. Malalignment of the back was decreased to the degree of twist shape and the head was aligned with the pelvis level *(Fig. 6)*. Nevertheless, the team will continue work in optimizing the prosthetic shoe, as many factors, i.e., materials with good quality of shock absorption and pressure distribution, should be taken into further consideration. Furthermore, intrinsic factors of the elephant should be concerned with rapid growth of the juvenile, thus bone density, blood calcium level monitoring and proper nutrition are required. Behavioral and emotional change, such as social behavior and sex interaction also should be observed closely.

Conflict of Interest

The authors declared no conflict of interest.

Acknowledgements

The authors would like to acknowledge the Faculty of Engineering, Chiang Mai University and the Rubber Authority of Thailand for establishment of the shoe prototype. We also acknowledge the Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University for equipment support and extend gratitude to M.L.Pipattanachart Diskul, D.V.M. Royal Elephant Stable for the valuable suggestions and advice.

Author Contributions

Conceptualization, S.K. and T.A.; methodology, S.K., K.W. and T.A.; software, K.W.; resources, W.L., K.N., B.C. and P.P.; writing-original draft preparation, S.K. and B.C.; writing-review and editing, T.A. and K.W.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 661-662, 2021
DOI: 10.9775/kvfd.2020.26478

LETTER TO THE EDITOR

Acute Interstitial Pneumonia in Nongrazing Cows [1]
(Merada Otlamayan İneklerde Akut İntersitisyel Pnömoni)

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[1] *This study was previously presented at 1st International Farm Animal Medicine Congress, 14-17 April 2019, Fethiye, Mugla, Turkey and 13rd National-2nd International Veterinary Internal Medicine Congress, 11-13 October 2019, Ankara, Turkey*

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Article ID: KVFD-2020-26478 Published Online: 27.09.2021

Dear Editor,

Acute interstitial pneumonia (AIP) (also known as atypical interstitial pneumonia, acute bovine pulmonary edema and emphysema, fog fever) is an uncommonly observed disease of feedlot cattle caused by the ruminal conversion of tryptophan to 3-methylindole after grazing at lush tryptophan-rich pasture [1-3]. This letter aims to describe cases of AIP observed in nongrazing lactating Holstein cows and to draw attention to this disease. Owner consent has been obtained for reporting the cases.

Atypical pneumonia was observed in 10 (aged between 3-8 years old) of 74 cows in early lactation over a period of 20 days in a herd of 162 cows. Increased respiratory rate (R=36-60/min), laboured and open-mouth breathing, and frothing around the mouth were seen in affected animals *(Fig. 1)*. Clinical signs were mild in one and moderate-severe in other animals. Fever (T=38.8-39.7°C) or coughing was not present. Animals also demonstrated reluctancy to walk and slow movements. Other cows in the early lactation period had decreased milk yields.

Pneumonia treatment was initiated with ceftiofur sodium (1.1 mg/kg, im, once per day, Seftivet®, Deva Holding AS, Istanbul, Turkey), but no improvement was observed. Serum samples obtained from affected animals revealed IBR positivity in one, and BVD, BRSV and PI3 seropositivity in another animal. Necropsy of the first dead cow revealed noncollapsing lungs bearing impressions of costae and widespread emphysema *(Fig. 2)*. Inconsistency of clinical signs with infection, ineffectiveness of antibiotics and observation of severe emphysema at necropsy suggested

Fig 1. Frothing around the mouth and open-mouth breathing in a cow with acute interstitial pneumonia

AIP. Detailed questioning revealed that the animals had been consuming 5 kg of alfalfa hay and in the last 1.5 months additionally 3 kg alfalfa silage daily. Feeding with silage was ceased immediately and treatment for AIP was started with dexamethasone (0.05 mg/kg, im, single dose, Devamed®, Topkim, Istanbul, Turkey) in addition to antibiotics. Clinical signs did not improve except for one animal with mild clinical signs and 9 of the 10 animals died within 2-5 days. Severe emphysema of the lungs was evident at necropsy of all dead animals.

How to cite this article?

Batmaz H, Cangül İT: Acute interstitial pneumonia in nongrazing cows. *Kafkas Univ Vet Fak Derg,* 27 (5): 661-662, 2021.
DOI: 10.9775/kvfd.2021.26478

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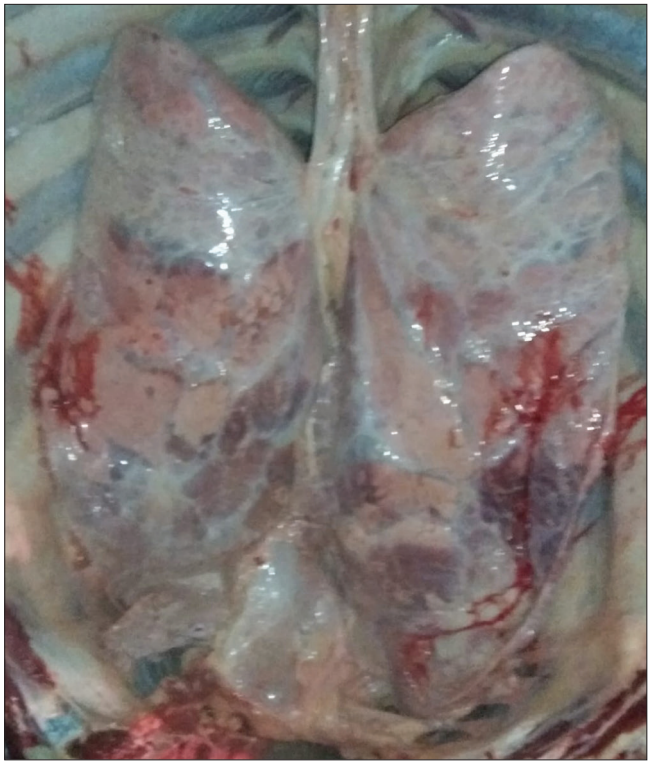

Fig 2. Emphysema in the lungs of the same cow at necropsy

Cessation of silage resulted in increased milk yield in cows at early lactation and no new cases were observed after 10 days. Alfalfa hay and alfalfa silage samples were analyzed with HPLC-FLD and tryptophan levels were measured as 225 mg/100 g (11.25 g from 5 kg) ve 153 mg/100 g (4.60 g from 3 kg), respectively. Animals were also being fed 22 kg corn silage (containing 3.7 g tryptophan), 12 kg concentrate feed (containing 27.3 g tryptophan), and 2 kg wheat bran (containing 4.9 g tryptophan) which summed up to ingestion of a total of 51.75 g tryptophan daily (personal communication with Dr. Hidir Gencoglu, DVM, on 26th July, 2021).

The cows in the early lactation had a milk yield of 35-40 l/day entailing a daily tryptophan intake of 35-40 g according to the CNCPS model (CNCPS for Cattle, version 6.5.5, Cornell University, Ithaca, NY, USA). The figures revealed that the affected animals had been receiving 11-16 g/day excessive tryptophan in the last 1.5 months.

AIP, a disease of cattle mainly grazing at lush pasture in the northern hemisphere countries, can also occasionally occur due to housing in moldy barns, or exposure to pollens or irritating gases or fumes [4]. Animals in our cases were housed in semi-open barns and, thus, were not exposed to molds or nocuous smokes. AIP has been reported to occur particularly in cattle > than 2-years-old and 4-15 days after introduction to lush pasture. In our case, affected animals were 3-8 years old, were housed in semi-open barns, and the disease occurred after feeding with alfalfa silage. All affected animals were among the cows in the early lactation period, which were fed alfalfa silage and higher amounts of carbohydrates to prevent negative energy balance. Tryptophan, particularly from lush plants, is quickly converted by *Lactobacillus* spp. in rumen to 3-methylindole, the major metabolite causing the disease and consumption of high amounts of carbohydrates increases the number of *Lactobacillus* spp. and, thus, is a contributing factor to the occurrence of the disease [4].

As a conclusion we would like to emphasize the importance of considering non-infectious etiologies in the differential diagnosis of pneumonia and that AIP may also occur in cows with no access to lush pastures. Levels of tryptophan should be taken into consideration when feeding roughage to lactating cows.

Acknowledgement

The authors have equally contributed to the preparation of this manuscript and has no conflicting financial interests.

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Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (5): 663-673, 2021
DOI: 10.9775/kvfd.2021.26113

REVIEW

Medicinal Plants Used in Wound Treatment in Veterinary Folklore in Turkey: A Literature Review

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Article ID: KVFD-2021-26113 Received: 06.06.2021 Accepted: 23.09.2021 Published Online: 24.09.2021

Abstract

Medicinal plants have been widely used as a source of medicine in treating human and animal diseases from past to present in Turkey, as in almost all cultures. In this review article, a comprehensive literature search was done, and the plants used to treat wounds in the folklore of veterinary medicine in Turkey were compiled. In total, it was determined that plants from 69 families were used. It was determined that the most commonly used plants belong to the families of Asteraceae (24), Rosaceae (16), Lamiaceae (13). It has been observed that the use of *Vitis* sp., *Pinus* sp., *Quercus* sp., and species such as *Rhus coriaria, Allium cepa, Olea europaea, Allium sativum* spread over vast geography throughout the country. At the end of our review, it can be said that some of the plants used in wound treatment are used in humans and animals in our country and in different countries, and other plants have the potential to create a source for new drugs to be developed in wound treatment.

Keywords: *Medicinal plants, Traditional treatment, Turkey, Veterinary folklore, Wound*

Türkiye'de Veteriner Hekimliği Folklorunda Yara Tedavisinde Kullanılan Tıbbi Bitkiler: Bir Literatür Değerlendirmesi

Öz

Tıbbi bitkiler, hemen hemen bütün kültürlerde olduğu gibi Türkiye'de de geçmişten günümüze insan ve hayvan hastalıklarının tedavisinde ilaç kaynağı olarak yaygın bir şekilde kullanılmaktadır. Bu derleme çalışması ile kapsamlı bir literatür araştırması yapılarak Türkiye'de veteriner hekimliği folklorunda yara tedavisinde kullanılan bitkiler derlenmiştir. Toplamda 69 Familyadan bitkinin kullanıldığı tespit edilmiştir. En yaygın kullanılan bitkilerin Asteraceae (24), Rosaceae (16), Lamiaceae (13) ailelerine ait olduğu belirlenmiştir. *Vitis* sp., *Pinus* sp., *Quercus* sp., cinslerinin ve *Rhus coriaria, Allium cepa, Olea europaea, Allium sativum* gibi türlerin kullanımının ise ülke genelinde geniş bir coğrafyaya yayıldığı görülmüştür. Yaptığımız derleme sonunda, yara tedavisinde kullanılan bitkilerin, bir kısmının ülkemizde ve farklı ülkelerde insan ve hayvanlarda kullanıldığı, diğer bitkilerin, yara tedavisinde geliştirilecek yeni ilaçlar için kaynak oluşturma potansiyeli taşıdığı söylenebilir.

Anahtar sözcükler: *Geleneksel tedavi, Tıbbi bitkiler, Türkiye, Veteriner folkloru, Yara*

INTRODUCTION

The main starting point of traditional folk veterinary medicine, ethnoveterinary medicine or veterinary folklore, involves the local system of knowledge, skills, practices and beliefs created by the people to protect the health and welfare of animals [1-3]. The content of this definition is comprehensive. However, research in recent years has focused on using traditional herbs to prevent, control, and treat animal diseases [4].

There has been an increasing interest in studies on medicinal plants and their local uses in different parts of the World, starting from the '80s all over the world. Documentation of traditional data through ethnobotanical studies is essential for conserving and using biological resources [5]. The fact that a plant is used to treat the same disease in different parts of the world is accepted as a proof of its pharmacological effect. Performing pharmacological studies on such plants and comparing the obtained information with laboratory studies provides a severe potential to drug research [6].

How to cite this article?

Çavuş Alan S, Özen R: Medicinal plants used in wound treatment in veterinary folklore in Turkey: A literature review. *Kafkas Univ Vet Fak Derg,* 27 (5): 663-673, 2021.
DOI: 10.9775/kvfd.2021.26113

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Wound therapy is a very ancient practice dating back to prehistoric times. Wound treatment is mentioned in the written sources in the Laws of Hammurabi in Babylon and the Smith and Ebers Papyrus in Ancient Egypt [7-9]. In the "Corpus Hippocraticum" in Ancient Greece, local drugs used against injuries, interventions and bandages applied are explained [8]. In ancient Rome, Celcus spoke of wound dressing and what to do in case of wounds [8,9]. However, an example of the first wound treatment is the attempt to heal the wounds of animals by licking them, which is thought to have started instinctively thousands of years ago [7].

This paper, it is aimed to create new and natural resources for drug development research in wound treatment today by compiling the traditional use of plants in wound treatment among the people in veterinary medicine in Turkey -comparing with their use in different geographies of the World.

Wound and Types of Wounds

The wound, which is the subject of the review article, is defined as the disruption of a tissue's cellular and anatomical integrity [10,11]. The wound healing process consists of a series of cellular and biochemical events that lead to the recovery of the injured tissue's strength and the restoration of its structural and functional integrity [10]. Wounds; although classified in various ways according to its causes, degrees, healing times, depth or skin integrity [12,13], are surgically examined in five main groups as cut wounds, sharp object wounds, and tear wounds, and open wounds. In addition to these, bite, poison and gunshot wounds can be counted [12].

Medical Plants Used for Wound Treatment in Animals in Turkey

In many countries of the world, plants are used to heal the wounds of animals, some of them can be effective and the accuracy of these effects is supported by scientific *in vivo* or *in vitro* studies [14]. Plants have been used for many years in the treatment of wounds among the people in Turkey. As a result of the detailed literature reviews, it was determined that 69 families of plants are used in the wound treatment of animals among the people in our country. Information about these plants is given in *Table 1*. It was determined that the most commonly used plants belong to the families of Asteraceae (24), Rosaceae (16), Lamiaceae (13). It has been observed that the use of *Vitis* sp., *Pinus* sp., *Quercus* sp., and species such as *Rhus coriaria, Allium cepa, Olea europaea, Allium sativum* spread over a wide geography.

Table 1. *Plants used in wound treatment in animals in Turkey**

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Adoxaceae	*Sambucus ebulus*	Sultanotu, Pıyran, Haptovina, Ademotu, Piran, Mülver, Mürver otu, Mürver ağacı, Sultanotu, Telligelin, Memer	Leaves, Aerial part, Fruit, Flower, Stem	Snake bite wound, Wound	[15-18]
	Sambucus nigra	Patpatik, Mürver	Leaves	Wound	[18,19]
Amaranthaceae	*Beta vulgaris*	Pancar, Şeker Pancarı, Şeker	Root	Wound, Wormy wound, Mouth wound (in Foot and Mouth Disease)	[20-24]
	Caroxylon articulatum	Banbal otu	-	Wound	[25,26]
Amaryllidaceae	*Allium sp*	Yabani soğan	Bulb	Bee and fly sting wound	[24,27]
	Allium ampeloprasum	Purasa	Leaves	Wound	[28-29]
	Allium cepa	Soğan	Bulb	Wound, Breast wound, Foot wound, Eye wound, Skin Wound	[21,26,28,30-41]
	Allium sativum	Sarımsak	Bulb	Wound, Mouth wound (in Foot and Mouth Disease), Skin wound (in sheep poxvirus, in Hypodermosis), Uterine wound (Metritis), Foot wound, Snake and scorpion bite wound, Bite wound, Sharp object wound, Nail wound, Eye wound	[20,22,24,26,27,29,31,36-43,44**,45-48]
Anacardiaceae	*Pistacia terebinthus*	Sakız ağacı, Menengiç	Resin	Wound	[35,49]
	Rhus coriaria	Sumak, Hamsıfto, Humsithso, Sımak, Sımmak, Tetre	Fruit, Seed, Resin, Leaves, Bunch	Mouth and Foot wound (in Foot and Mouth Disease), Cut wound	[20,21,23,31,35,44,49-58]
Apiaceae	*Apium graveolens*	Kereviz	Leaves	Wormy wound	[45]
	Coriandrum sativum	Kişniş	Seed	Wound	[38,39]
	Cuminum cyminum	Kimyon	-	Wound	[37,59]
	Eryngium campestre	Boğa dikeni, Yel kovdu dikeni	Root, Leaves, Flower	Snake bite wound	[60]
	Ferula orientalis	Çağşır, Kinkor	Root	Wound (in Foot and Mouth Disease), Wormy wound	[61,62]
	Ferula rigidula	Çakşır, Çağşır	Root	Skin wound	[31]
Apocynaceae	*Nerium oleander L.*	Zakkum, Ağı çiçeği, Yetim çiçeği, Ağu ağacı	Leaves, Flower	Wound, Saddle wound	[24,51]
Arecaceae	*Phoenix dactylifera*	Hurma	Fruit	Wound, Old wound	[27,36,45,48]

Table 1. *Plants used in wound treatment in animals in Turkey* (continued...)*

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Araceae	*Arum* sp.	Kari, Kardi	Root	Wound	[35]
	Arum maculatum	Yılandili otu	Aerial parts	Wound	[22]
Aristolochiaceae	*Aristolochia* sp.	Loğusa otu	Root	Wound	[45]
	Aristolochia bottae	Lohusa otu, Zılındar, Guhê gur, Guhok Helkêşılkat, Guhkıtık, Hakkutık, Zelindar, Zirindar	Root, Entire plant, Fruit	Wound, Horn area wound, Foot sole wound, Purulent wound	[22,50,61,63-66]
	Aristolochia maurorum	Gavur bostanı	Fruit	Wound	[67]
Asparagaceae	*Ornithogalum pyrenaicum*	İt soğanı	Fruit	Wound	[19]
	Ornithogalum umbellatum	Akyıldız, Sunbala, Köpek soğanı, ükrükotu	Root	Wound	[17]
Asteraceae	*Achillea biebersteinii*	Sarı civanperçemi	Entire plant	Foot wound (in Foot and Mouth Disease)	[68]
	Achillea millefolium	Beyaz civanperçemi	Entire plant	Foot wound (in Foot and Mouth Disease)	[68]
	Achillea wilhelmsii	Sarı civanperçemi	Entire plant	Foot wound (in Foot and Mouth Disease)	[68]
	Anthemis chia	Beyaz papatya, Eşek papatyası, Papatya	Fruit	Wound, Foot wound	[17,40,69]
	Arctium minus	Gurn, Kabalak, Pıtrak	Leaves	Purulent wound	[19,70]
	Arctium tomentosun	Dulavrat otu, Kelotu	Root, Flower, Leaves	Wound	[17]
	Artemisia absinthium	Acı yavşan	Aerial parts	Wormy wound	[71]
	Aster L.	Papatya	Aerial parts	Wound, Mouth wound	[41]
	Carlina corymbosa	Kırkbaş dikeni	Aerial parts	Wound	[72]
	Carlina gummifera	Deve dikeni	Aerial parts	Wound	[30]
	Carthamus tinctorius	Usfür	Stem	Wound	[45]
	Centaurea glastifolia	Tahliş	Aerial parts	Purulent wound	[64]
	Centaurea karduchorum	Güya brınok	Aerial parts	Purulent wound	[64]
	Centaurea pterocaula	Tahliş	Aerial parts	Purulent wound	[64]
Betulaceae	*Corylus L.*	Fındık	Oil	Skin wound	[41]
Boraginaceae	*Alkanna tinctoria*	Havacıva otu	Root	Skin wound	[31]
	Anchusa azurea	Sığırdili, Gurüz, Gürüz, Tort, Güriz, Guriz, Hımhım, Geriz	Leaves, Aerial parts	Wound, Snake bite wound, Wormy wound	[22,23,30,35,75]
Brassicaceae	*Brassica oleracea*	Karalahana, Lahana	Leaves	Wound, Sunburn wound	[20,29,76]
	Capsella bursa – pastoris	Övez otu, Tahtacı otu, Muska, Muska otu, Tuzluca, Tuzluca otu	Leaves	Wound	[77]
	Cardaria draba	Acıkavuk	Leaves	Head area wounds	[78]
	Isatis tinctoria	Çivitotu	Leaves	Mouth wound	[41]
	Sinapis alba	Beyaz hardal, Hardal	Seed	Wound	[24,28,45,47]
Campanulaceae	*Campanula glomerata*	Nojda	Leaves	Purulent wound	[64]
	Campanula involucrata	Nojda	Leaves	Purulent wound	[64]
Cannabaceae	*Cannabis sp*	Kendir	-	Wound	[24,28]
Caprifoliaceae	*Dipsacus laciniatus*	Eşekkengeri	Aerial parts	Mouth wound	[74]
Caryophyllaceae	*Dianthus* sp.	Karanfil	Flower	Wound, Mouth wound	[38,39]
	Gypsophila arrostii	Çöğen otu	-	Wound	[45]
	Gypsophila struthium	Voynik otu	-	Wound	[45]
	Myrtus communis	Mersin	Leaves	Wound	[25,26,37-39,47]
Cladophoraceae	*Cladophora glomerata*	Yosun	Leaves	Wound	[21]
Combretaceae	*Terminalia citrina*	Helile, Sarı helile	Core	Wound	[28,38,39]
Convolvulaceae	*Convolvulus arvensis L*	Çoban döşeği otu	Leaves	Wound	[21]
	Convolvulus scammonia	Bingöz otu	-	Wound	[47]
Cornaceae	*Cornus mas*	Kızılcık, kiren	Fruit	Mouth wound (in Foot and Mouth Disease)	[79]
	Cornus sanguine	Kızılcık, kiren	Fruit	Mouth wound (in Foot and Mouth Disease)	[79]

Table 1. *Plants used in wound treatment in animals in Turkey* (continued...)*

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Cucurbitaceae	*Citrullus colocynthis*	Ebu Cehl karpuzu	Fruit	Wound, Nail crack	[28,45]
	Citrullus lanatus	Karpuz	Fruit	Mouth wound (in Foot and Mouth Disease)	[80]
	Cucurbita sp.	Kabak	Fruit	Wound	[38,39,49]
	Cucurbita mixta	Kış kabağı	Fruit	Purulent wound	[35]
	Ecballium elaterium	Eşek hıyarı, Cırtlatan otu, Acı dülek, Acı kavun	Aerial parts, Root	Skin wound	[17,31]
	Lagenaria siceraria	Su kabağı	Fruit	Bite wound	[45]
Cupressaceae	*Cupressus L.*	Servi	Leaves	Wormy wound	[45]
	Juniperus drupacea	Andız, Andız ardıcı	Stem	Wound	[17,40]
	Juniperus oxycedrus	Ardıç, Dikenli ardıç, Katran ardıcı	Tar, Branch, Pine cone, Resin	Wound, Breast wound, Snake bite wound, Foot wound	[25,35,37,41,47,56,70,81-85]
Cyperaceae	*Cyperus rotundus*	Topalak	Oil	Snake bite wound	[45]
Ericaceae	*Arbutus andrachne*	Kızıl sandal	-	Wound	[24]
	Erica arborea L	Piren, Püren	Fruit	Mouth and foot wound (in Foot and Mouth Disease)	[18]
Euphorbiaceae	*Euphorbia* sp.	Sütlük, Sütleğen otu	Milk	Wound, Snake bite wound, Foot wound (in Foot and Mouth Disease)	[25,86,87]
	Euphorbia kotschyana	Sütlük, Sütleğen	Milk, Aerial parts	Wormy wound	[71,88]
	Euphorbia macroclada	Sütleğen otu	Milk	Wound	[21]
Fabaceae	*Astragalus* sp.	Guni, Anzerut, Anzarut, Anzarot, Anzorut	Aerial parts	Wound, Foot wound (in Foot and Mouth Disease)	[23,25,28,38-40,48,85]
	Cicer arietinum	Nohut	Seed	Wound	[28,37]
	Glycyrrhiza glabra	Meyan kökü	Root	Mouth wound	[27,41]
	Lathyrus sp	Maş otu, Mürdümük	-	Wound	[28,29]
	Lens culinaris	Mercimek	Seed	Wound, Mouth wound	[27,40,59,89]
	Medicago sativa	Kaba yonca, Çevrince	Aerial parts	Wound	[17]
	Phaseolus vulgaris	Fasulye	Aerial parts	Wound	[30]
	Trifolium repens	Nefel	Aerial parts	Wormy wound	[64]
	Trigonella foenum-graecum	Çemen otu	-	Old wound, Nail crack	[36,45]
	Vicia ervilia	Burçak	-	Wound	[40,45 76,85]
	Vicia faba	Bakla, delice bakla	Seed, Fruit	Wound	[17,48]
Fagaceae	*Quercus* sp.	Meşe, Palamut, Mazı, Kerçik, Şakalor (gal), Pelit	Leaves, Ash, Bark, Fruit, Root	Wound, Laminitis, Wormy wound, Breast wound, Foot wound (in Foot and Mouth Disease), Back wound, Abdminal wound	[20,22,24,25,27,28,30,35-39,41,45,47,49,59]
Hypericaceae	*Hypericum* sp.	Bahtof, Batof, Batuf, Botav	Entire plant, Branch, Flower	Wound	[23]
	Hypericum perforatum	Kantaron, ilaç otu, Kantiran otu, Yara otu, Binbirdelikotu, Kantaron, Kangran, Kantıron	Leaves, Flower, Branch, Oil	Back wound, Sharp object wound, Foot wound (in Dermatitsi Madidans), Skin wound, Breast wound, Cut wound	[17,41,54,58,59,76,90,91]
Iridaceae	*İris germanica*	Süsen	Root	Wound	[29]
	Nepeta nuda L.	Nojda	Aerial parts	Purulent wound	[64]
	Origanum majorana	Guy otu, Mercanköşk, Akkekik	Flower	Wound	[17]
	Origanum onites	Kekik, Salman kekik, İncir kekiği	Leafy branch	Mouth wound (in Foot and Mouth Disease)	[92]
	Salvia macrochlamys	Bareş, çirçirik	Aerial parts	Wormy wound	[64]
	Salvia verticillata	Bareş, Emme çiçeği, Şalba, Kara şalba	Aerial parts	Wound, Wormy wound	[60,64,93
	Scutellaria orientalis	Kesel mahmuda şin	Aerial parts	Wormy wound	[64]
	Stachys balansae	Bareş	Aerial parts	Purulent wound	[64]
	Stachys kurdica	Bareşa kulikzer	Aerial parts	Purulent wound	[64]
	Thymbra spicata	Zahter, Kara kekik	Stem, Leaves,	Wound	[17]
	Thymus cilicicus	Kekik, Yer kekiği, Kılçık kekiği	Aerial parts	Wound	[17]
Juglandaceae	*Juglans* sp.	Ceviz	Bark, Leaves, Fruit	Foot and Mouth wound (in Foot and Mouth Disease)	[26,39,41,47,54]
	Juglans regia L.	Ceviz	Unripe fruit	Wound	[94]
Labiatae	*Calamintha nepeta subsp. Glandulosa*	Yılan otu	Root, Aerial parts	Snake bite wound	[18]

Table 1. *Plants used in wound treatment in animals in Turkey* (continued...)*

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Lamiaceae	*Lavandula stoechas*	Karabaş otu	Aerial parts	Wound	[17]
	Mentha sp.	Yabani nane, pung, Punk, Yarpuz, Kara nane, Yarpız	Leaves, Aerial part	Wound, Skin wound	[22,26,35,41]
	Ocimum basilicum	Reyhan	Aerial parts	Mouth wound (in Foot and Mouth Disease)	[44]
	Rosmarinus officinalis	Biberiye, Beyaz püren, Kuşdili	Flower, Leaves	Wound	[17]
	Salvia argentea	Beyaz şabla	Aerial parts	Wound	[95]
	Salvia fruticosa	Adaçayı, Boş, Boşotu, Boşapla, Muşapla, Moşapla, Puşapla, Şapla, yakıotu	Aerial parts	Breast wound, Genital area wound	[95]
	Salvia tomentosa	Adaçayı, Muşapla, Calba, Çalba otu, Çalba Bitkisi, Çalba, Can taze, Boşşapla, Boşçapla	Aerial parts	Wound, Breast wound	[95-97]
	Salvia virgata	Fatmana otu, Yılancık	Leaves	Wound	[17]
	Stachys lavandulifolia	Bareş	Aerial parts	Wormy wound	[64]
	Teucrium polium	Giya teşenek, Giya mervend	Flower, Root, Leaves	Wound	[65]
	Thymus sp.	Kekik	Aerial parts	Foot and Mouth wound (in Foot and Mouth Disease), Skin wound	[41,44,54,76,98-100]
	Thymus zygioides	Bayır çayı, Kaya kekiği, Kekik, Kır Kekiği, Nuzla otu, Şeker otu, Taş kekiği, Toğga	Aerial parts	Snake bite wound	[95]
	Vitex agnus-castus	Kürf	Aerial parts	Foot wound	[49]
Lauraceae	*Laurus nobilis*	Defne, Tefrün, Tehnel	Fruit, Leaves, Seed	Wound, Rectal wound	[17,25,31,47]
	Laurus camphora	Kafur	-	Breat wound, Nail crack	[45]
Liliaceae	*Lilium sp*	Zambak	Seed	Wound	[27]
	Ruscus aculeatus	Tavşan memesi	Root	Wound	[17]
	Tulipa armena	Dağ lalesi, Lale	Fruit	Wound	[19]
Linaceae	*Linum nodiflorum*	Yaban, Keten	Seed	Wound	[17]
	Linum usitatissimum	Keten tohumu, Bezir	Seed	Wound	[39,40,45,47]
Lythraceae	*Lawsonia inermis*	Kına, Hınna	Leaves	Wound, Skin wound (in Trichophyte), Ulcer (in Lymphangitis), Foot wound	[20,23,25,26,37-40,45,47,54,85]
	Punica granatum	Nar	Bark, Fruit, Flower	Wound, Foot wound (in Foot and Mouth Disease), Mouth wound	[25,27, 28,31,45,48,58,59, 101***, 102]
Malvaceae	*Alcea* sp.	Hiro otu, Hero, Hatmi	Root, Entire plant, Flower	Wound, Mouth wound	[22,23,25,35,36,38-40,69, 103]
	Gossypium herbaceum	Pamuk, Panbuk	Seed	Wound	[25,28,45,103]
	Malva neglecta	Ebegümeci Dolık, Dolik, Dollık, Tolık, Küçük ebegümeci, Çoban çöreği, Tolik, Tollik, Ebegömeci	Leaves, Aerial parts	Wound	[17,30,35,49]
Moraceae	*Ficus carica*	İncir	Milk, Fruit, Leaves	Skin wound, Foot wound, Nail splitting	[25,26,28,31,37,40]
	Morus sp.	Dut	Molasses, Marmalade	Wound, Mouth wound	[23,30,49,104,105]
	Morus nigra	Karadut	Fruit	Mouth wound	[41,71]
Nitrariaceae	*Peganum harmala*	Harmel, Üzerlik	Seed	Snake and scorpion bite wound	[28, 29, 103]
Oleaceae	*Jasminum* sp.	Yasemin	Oil	Wound	[24]
	Olea europaea	Zeytin	Oil, Leaves, Core	Wormy wound, Skin wound, Foot sole wound, Back wound, Sunburn wound, Cut wound, Foot wound, Breast wound, Mouth wound (in Foot and Mouth Disease), Nail crack	[20,23,25,27-29,31,32,34,37-41,43-47,49, 54,56,76,85,102,106-108]
Orobanchaceae	*Euphrasia pectinata*	Göz otu	-	Wound, Foot wound	[47]
Papaveraceae	*Fumaria officinalis*	Şahtere otu	-	Wound	[38,39]
	Papaver rhoeas	Gelincik, Şkayyek, Edorak, Adorak	Fruit, Seed, Flower	Wound	[17,35]
	Papaver somniferum	Haşhaş	Oil	Wound	[105]
Pedaliaceae	*Sesamum indicum*	Susam, Tahin	Seed	Wound, Burn wound, Back wound, Breast wound, Foot wound	[25,28,32,36,47,56,76,103]
Pinaceae	*Abies* sp.	Köknar	Resin	Foot wound (in Foot and Mouth Disease)	[106]

Table 1. *Plants used in wound treatment in animals in Turkey* (continued...)*

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Pinaceae	*Cedrus libani*	Sedir ağacı, Sarı Katran, Ardıç	Tar, Branch, Stem, Leaves	Wound, Head area wound (in Actinomycosis), Wormy wound, Mouth wound (in Foot and Mouth Disease), Foot wound, Skin wound, Breast wound, Snake and scorpion bite wound	[36,58,71,80,109,110]
	Picea sp.	Ladin	Resin, Extract	Wound, Cut wound	[41,106]
	Pinus sp.	Çam	Resin, Tar, Oil, Bark, Püse	Foot wound (in Foot and Mouth Disease), Foot wound (İn Dermatitis Madidans), Sharp object wound, Sunburn wound, Skin wound (in Trichophyte, in Hypodermosis), Operation wound (in Castration), Mouth wound (in Foot and Mouth Disease), Wormy wound, Cut Wound, Purulent wound, Breast wound, Nail crack	[20,25,26,28,37-41,43,47,48,54,56,80,83-86,100,106,107,109,111-116]
	Pinus brutia	Kızılçam, Çam, Sakız çamı, İşam, Gara Şam, Gızılşam, Kızılşam	Tar, Resin	Wound, Breast wound	[32,72,81,88,98,117]
	Pinus nigra	Karaçam, Garaçam, Katran çamı, Karasakız, Fıstık çamı	Tar, Resin, Bark, Wood	Wound, Mouth wound, Foot wound, Skin wound (İn Hypodermosis)	[21,33,41,79,104,118,119]
	Pinus sylvestris	Sarıçam	Tar	Wound	[79]
Piperaceae	*Piper nigrum*	Karabiber	-	Snake bite wound	[45]
Plantaginaceae	*Plantago* sp.	Sinirli ot, Bağa, Pel heves otu, Peli heves Damalca, Sinir otu, Hilan dilan, Berghewes, Pelevez	Aerial parts, Leaves	Wound, Purulent wound, Bee sting	[20,22,23,30,35,49,103]
	Plantago lanceolata	Gıyamambel, Gıyabirinok, Belgpanık, Sinir otu, Damar otu, Bağaotu, Bağa yaprağı, Damarlıca, Yılandili, Sinirliot, Çıbanotu, Sivrice, Sivrisilik	Leaves	Wound, Purulent wound, Skin wound (in Trichophyte)	[17,21,41,61,64,97]
	Plantago major	Pelhewes, Sinir otu	Leaves	Nail wound, Purulent wound	[62,96]
Plumbaginaceae	*Acantholimon acerosum*	Geven, Çobanyastığı	Root	Wound	[19]
	Acantholimon armenum	Geven, Yılanyatağı	Root	Purulent wound	[19]
Poaceae	*Hordeum* sp.	Arpa	Aerial parts	Wound, Mouth wound (in Foot and Mouth Disease)	[23,28,48,116]
	Oryza sp.	Pirinç	Seed	Wound	[76]
	Secale cereale	Çavdar	-	Wound	[106]
Poaceae	*Triticum sp.*	Buğday, Kepek	Aerial parts	Wound, Nail wound, Foot wound	[23,25,28,34,36,38-41,47,69,85]
	Zea mays	Mısır	Fruit	Wound	[23]
Polygonaceae	*Rheum ribes*	Işkın, Işgın, Rivez	Stem, Root	Wound, Mouth wound (in Foot and Mouth Disease)	[30,98,99]
	Rumex sp.	Tırşik, Tırşok, Kuzukulağı	Aerial parts	Wound	[30]
	Rumex acetosella	Kuzukulağı, Tırşok Tırşik	Aerial parts	Wound	[30]
Portulacaceae	*Portulaca oleracea*	Semizotu	Aerial parts	Wound, Wound (in Fistulous withers), Eye wound	[24,27,28,36,41]
Ranunculaceae	*Delphinium staphisagria*	Bit otu	Seed	Wormy wound	[54]
	Helleborus orientalis	Karacaotu, Karaot, Kocaot	Root	Wound, Purulent wound	[15,38,39,47]
	Nigella sp.	Çörek otu	Seed	Mouth wound, Nail splitting	[26,27,36,38,39]
Rhamnaceae	*Paliurus spina-christi P. Mill.*	Karaçalı	Seed	Wound	[41]
Rosaceae	*Agrimonia eupatoria*	Fıtık otu, Kızıl otu, Kızıl yaprak	Aerial parts	Wound	[17]
	Amygdalus communis	Badem, Badem ağacı	Fruit, Oil, Root	Wound	[24,35,36,40,103]
	Crataegus monogyna	Yemişgen çalısı	Thorn	Snake bite wound	[120]
	Cydonia oblonga	Ayva	Core	Breast wound, Mouth wound	[42,59]
	Fragaria vesca	Yelbezen çileği, yabani çilek	Leaves	Wound	[41]
	Malus pumila	Elma	Fruit, Leaves	Wound, Mouth wound (in Foot and Mouth Disease), Snake bite wound, Foot wound	[21,41,84,102]
	Persica vulgaris	Şeftali (tüylü)	Leaves	Skin wound	[121]

Table 1. *Plants used in wound treatment in animals in Turkey* (continued...)*

Family	Plant Species	Vernacular Name	Part(s) Used	Purpose of Usage	References
Rosaceae	*Prunus* sp.	Erik	Resin, Molasses	Wound, Nail crack	[38,39,41,49]
	Prunus armeniaca	Kayısı	Resin	Wound	[49]
	Prunus divaricata Ledeb.	Yunuseriği	Fruit	Wound	[56]
	Prunus laurocerasus	Taflan ağacı	Leaves	Foot wound (in Foot and Mouth Disease)	[41]
	Prunus mahaleb	Mahlep	Seed	Wound	[28]
	Prunus persica	Şeftali	Leaves	Wound, Skin wound, Foot wound (in Foot and Mouth Disease), Wormy wound, Back wound	[21,28,30,31,51,106]
	Prunus spinosa	Çakaleriği, Erik, Dağ eriği, Domuz eriği, Yabani erik	Fruit, Resin, Stem	Wound, Mouth wound, Breast wound	[56,115,122]
	Rosa sp.	Gül	Oil	Wound, Breast wound, Burn wound	[24,27,31,36,40,45,47,103]
	Rubus discolor	Böğürtlen	Leaves, Root	Wound, Foot wound	[25,30,35]
Rubiaceae	*Galium verum*	Boyalık, Yoğurtotu, Iplicik	Aerial parts	Wound	[17]
Rutaceae	*Citrus limon*	Limon	Fruit	Wound, Foot and mouth wound (in Foot and Mouth Disease), Eye wound	[21,22,31,41,36,43,83]
Salicaceae	*Salix* sp.	Söğüt ağacı	Ash, Bark	Wound	[25,38,39,47,85,102]
Scrophulariaceae	*Verbascum* sp.	Çalba, Sığırkuyruğu	Aerial parts	Wound	[25,86]
	Verbascum asperuloides	Sığırkuyruğu, Yalangı otu, Maçyanık, Yalanki	Leaves, Flower	Wound	[123]
	Verbascum cheiranthifolium	Sığırkuyruğu, kurtkulağı	Leaves, Flower	Wound	[20,21]
Solanaceae	*Capsicum* sp.	Biber, Büber	Fruit	Wound, Snake bite wound	[25,28,37,102]
	Capsicum annuum	Pul biber, Kırmızı biber	Fruit	Mouth wound	[35,49,55,100]
	Hyoscyamus sp.	Benc otu	-	Wound	[28]
	Nicotiana sp.	Tütün, Titün, Tütin	Leaves	Wound	[23,35,49]
	Solanum lycopersicum	Domates	Fruit	Head area wound (in Actinomycosis), Skin wound (in sheep poxvirus), Foot and mouth wound (in Foot and Mouth Disease), Burn wound, Eye wound	[20,22,30,31,34,35,41,49,76,84,98,118]
Tamaricaceae	*Tamarix* sp.	Ilgın ağacı	Ash	Wound	[24]
Teaceae	*Camellia* sp.	Çay	Leaves	Eye wound	[41]
Thymelaeaceae	*Daphne oleoides*	Çobansüpürgesi	Aerial parts	Wound	[19]
Ulmaceae	*Ulmus minor*	Karaağaç	Bark, Root	Wound	[15,18]
Urticaceae	*Urtica* sp	Isırgan, Isırgan otu, Küçük ısırgan ot, Tatlı ısırgan	Leaves, Root, Stem, Seed	Wound, Foot wound	[17,30,40,41,69]
Vitaceae	*Vitis* sp.	Asma, Üzüm, Tevek	Molasses, Leaves, Fuit, Root	Wound, Foot and Mouth wound (in Foot and Mouth Disease), Burn wound, Back wound, Foot wound (In Dermatitis Madidans), Snake and scorpion bite wound, Laminitis, Nail crack, Bite wound, Breast wound, Bee sting, Fly bite wound	[20,23-25,27-30,34-40,43,45-48,54,55,59,69,76,80,84,85,98,99,102,103,107,108,118]
Violaceae	*Viola* sp.	Menekşe	Oil	Nail crack, Breast wound	[27,40,45]
Xanthorrhoeaceae	*Eremurus spectabilis*	Çiriş	-	Wound	[25]
	Aloe sp.	Azvay	-	Wound	[24]
Zingiberaceae	*Curcuma longa*	Zerdeçal	-	Wound, Sharp object wound	[36]
	Zingiber officinale	Zencefil	-	Wound	[47]

** Publications from theses were not included in the article to avoid duplication. The family and species names of the plants are included in the table in their original form, based on the author's statement. In the studies where only the names of the plants are given and the names of the families and species are not written, the Turkish Plants List database was used while transferring them to the table*
*** The author reported that Allium sativum (garlic) belongs to the Liliaceae family. However, in today's classification, it is in the Amaryllidaceae Family*
**** The author reported that Punica granatum (pomegranate) belongs to the Punicaceae family. However, in today's classification, it is in the Lythraceae Family*

Conclusion

Today, despite the technological development in the pharmaceutical industry and the diversity of synthetic drugs, some plants are used by the public to treat animal diseases. Studies show that plants are a common alternative for the treatment of animal diseases in Turkey, originating from various reasons [20,22,23,30,31,35,41,49,76,80,98]. In parallel with this, when the recent clinical studies are examined, it is seen that the majority of the surgical diseases, but the studies on the healing of the wound in most diseases have gained weight [124,125] and the researchers have tried especially herbal treatment methods on the wound [126-129].

When *Table 1* is examined, the plants used in wound treatment are *Achillea millefolium* [3,130-135], *Alkanna tinctoria* [136], *Allium cepa* [133,137,138], *Allium sativum* [133,139,140], *Althaea officinalis* [133], *Artemisia absinthium* [133], *Brassica oleracea* [133,135], *Capsella bursa - pastoris* [130], *Curcuma longa* [138,140,141], *Ecballium elaterium* [133], *Hypericum perforatum* [3,130,131,133-135,142-147], *Juglans regia* [133,143,147], *Juniperus oxycedrus* [133], *Linum usitatissimum* [144], *Malva neglecta* [135,143,144], *Morus nigra* [133], *Myrtus communis* [138], *Nerium oleander* [133,137,140], *Olea europaea* [133,137,148], *Papaver rhoeas* [133], *Peganum harmala* [137], *Pistacia lentiscus* [148], *Plantago lanceolata* [135], *Plantago major* [131,133,145,146], *Prunus persica* [4,132,141], *Prunus spinosa* [148], *Punica granulatum* [137], *Rosmarinus officinalis* [142], *Sambucus ebulus* [133], *Sambulus nigra* [131-133,144,147,148], *Secale cereale* [131], *Teuchium polium* [137], *Ulmus minör* [133], *Urtica dioica* [132,133], *Zea may* [131,133] species and *Euphorbia* sp.[133], *Mentha* sp.[133,137], *Nicotina* sp.[139], *Rumex* sp.[138], *Salix* sp.[133], *Triticum* sp.[133,137], *Quercus* sp.[133], *Verbascum* sp.[133], *Vitis* sp.[133] have been found to be similarly used in wound treatment of animals in various countries of the World. The fact that these plants are used in wound healing in a wide geography from Italy to Canada, Spain, Algeria, Serbia, Romania, Pakistan, India, South Africa and South Asia may suggest that people have difficulty in accessing veterinary services or do not demand them for various reasons, and it also suggests that they prefer traditional treatment methods instead of these services.

Achillea millefolium [149-152], *Allium cepa* [149,153,154], *Artemisia absinthium* [152-154], *Capsella bursa - pastoris* [150], *Centaurea pterocaula* [149], *Curcuma longa* [10], *Cydonia oblonga* [154], *Ficus carica* [155,156], *Hypericum perforatum* [10,150,157-159], *Juglans regia* [152,156,160], *Lawsonia inermis* [5], *Malva neglecta* [62,149,161], *Olea europaea* [152], *Papaver rhoeas* [162], *Pinus nigra* [151], *Plantago lanceolata* [62,156,159,160], *Plantago major* [62,149,151,154-156,159,160], *Pinus sylvestris* [149], *Prunus armeniaca* [154], *Prunus persica* [154,163], *Rhus coriaria* [101,161], *Sambucus ebulus* [154,157], *Sambulus nigra* [154], *Urtica dioica* [158,160], *Vitis vinifera* [154], species and *Nicotina* sp.[154,164], *Pistacia* sp.[155], *Quercus* sp.[158], *Verbascum* sp.[158] genus are also used in folk medicine in wound treatment in various geographies of Turkey and the World. It can be argued that this situation is a concrete indication of the knowledge that human beings use plants for the treatment of their own diseases and also use them for the treatment of animals, which is thought to have existed since ancient times.

At the end of our review, it can be said that some of the plants used in wound treatment are used in humans and animals in our country and in different countries, and other plants have the potential to create a source for new drugs to be developed in wound treatment.

Conflict of Interest

All authors declare that there is no potential conflict of interest.

Author Contributions

All authors contributed to the content and main topics of the manuscript. The structure of this article was designed by S. Çavuş Alan and R. Özen, the literature was reviewed and the first version of the manuscript was prepared by S. Çavuş Alan and R. Özen.

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