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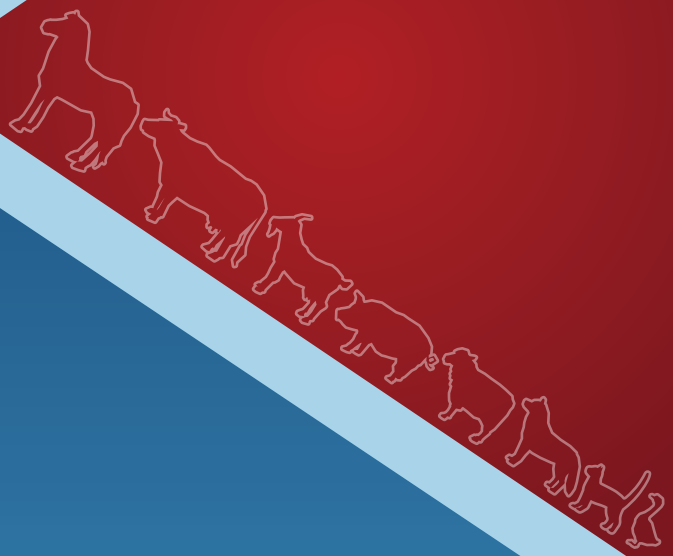

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RESEARCH ARTICLE

The Effect of Total Mix Ration with Xylitol Supplementation on *In Vitro* Ruminal Total Gas and Methane Production, Digestion Values, Organic Acids, Ammonia-Nitrogen, and the Number of Total Protozoa in Dairy Cattle

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Abstract: The study aimed to determine the effect of xylitol, added to the dry period total mix ration (TMR) of dairy cattle, on *in vitro* rumen fermentation, total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen contents and the number of total protozoa. Xylitol was added to dairy cattle TMR at different rates (0%, 2% 4%, and 8%; dry matter basis). The xylitol supplementations at 2 and 4% did not affect *in vitro* total gas production, *in vitro* methane production, metabolizable energy (ME) and organic matter digestibility (OMd) values (P>0.05). However, 8% xylitol supplementation decreased *in vitro* total gas production, *in vitro* methane production, ME and OMd (P<0.05). The molarities of total volatile fatty acid (TVFA) percentages of acetic acid (AA), propionic acid (PA) and butyric acid (BA) in TVFA, ammonia-nitrogen concentration, and the number of total ciliate protozoa of the *in vitro* rumen fluid of xylitol supplementations at 2%, 4%, and 8% were similar to those of control TMR (P>0.05). The iso-valeric acid (IVA), iso-butyric acid (IBA) and valeric acid (VA) percentages in TVFA of *in vitro* rumen fluid linearly decreased with xylitol supplementation, especially 8% xylitol supplementation (P<0.05). Besides, 2% and 4% xylitol supplementations to dairy cattle TMR numerically increased the concentration of ammonia-nitrogen and the number of total ciliate protozoa in *in vitro* fermentation fluid (P>0.05). Consequently, the supplementation of 2% and 4% xylitol to dairy cattle ration did not affect the *in vitro* rumen fermentation (total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen parameters). However, 8% supplementation xylitol to dairy cattle ration had the potential to affect the before-mentioned *in vitro* ruminal fermentation parameters adversely.

Keywords: *Dairy cattle, In vitro gas production, Rumen fermentation, Xylitol*

Ksilitol İçeren Süt Sığırı Karma Rasyonunun *İn vitro* Ruminal Total Gaz ve Metan Üretimi, Sindirim Değerleri, Organik Asit ve Amonyak-Azotu Parametreleri İle Total Protozoa Sayısına Etkisi

Öz: Çalışmanın amacı, süt sığırlarının kuru dönem toplam karma rasyonuna (TKR) ilave edilen ksilitolün *in vitro* rumen fermantasyonu, toplam gaz üretimi, metan üretimi, tahmini sindirim değerleri, organik asitler ve amonyak-azotu içerikleri ve toplam protozoa sayısı üzerindeki etkisini belirlemektir. Ksilitol, süt sığırı TKR'sinin kuru maddesine (KM) farklı oranlarda (%0, %2, %4 ve %8) ilave edilmiştir. Süt sığırlarının kuru dönem TKR'sine %2 ve %4 ksilitol ilavesi, kontrol TKR'sine göre *in vitro* toplam gaz üretimi, *in vitro* metan üretimi, metabolik enerji (ME) ve organik madde sindirimi (OMd) değerlerini değiştirmemiştir (P>0.05). Bununla birlikte, %8'lik ksilitol ilavesi *in vitro* toplam gaz üretimi, *in vitro* metan üretimini, ME ve OMd değerlerini azaltmıştır (P<0.05). Süt sığırlarının kuru dönem TKR'sine (KM'de) %2, %4 ve %8 ksilitol ilavesi *in vitro* rumen sıvısının toplam uçucu yağ asiti (TUYA) molaritesi, TUYA içindeki asetik asit (AA), propiyonik asit (PA) ve bütirik asit (BA) oranı, amonyak-azotu konsantrasyonu ve toplam siliatalı protozoa sayısı, kontrol TKR'sinin değerleriyle benzer olduğu belirlenmiştir (P>0.05). *In vitro* rumen sıvısının TUYA'deki iso-valerik asit (IVA), iso-bütirik asit (IBA) ve valerik asit (VA) oranları ksilitol ilavesiyle (özelllikle %8 ksilitolde) linear olarak azalmıştır (P<0.05). Ayrıca, süt sığırı TKR'sine %2 ve %4 ksilitol ilavesi, *in vitro* fermantasyon sıvısının amonyak-azot konsantrasyonunu ve toplam siliat protozoa sayısını rakamsal olarak artırdığı gözlenmiştir (P>0.05). Sonuç olarak, süt sığırı rasyonlarına %2 ve %4 ksilitol ilavesinin *in vitro* rumen fermantasyonunu (toplam gaz üretimi, metan üretimi, tahmini sindirim değerleri, organik asitler ve amonyak-azot parametreleri) etkilememiştir. Bununla birlikte, süt sığırı rasyonlarına %8 ksilitol ilavesinin, daha önce bahsedilen *in vitro* ruminal fermentasyon parametrelerini olumsuz etkileme potansiyeline sahip olduğu belirlenmiştir.

Anahtar sözcükler: *Süt sığırı, In vitro gaz üretimi, Rumen fermentasyonu, Ksilitol*

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Introduction

The transition period is the most critical physiological stage in dairy cattle health, production, reproduction, and profitability three weeks before and three weeks after parturition. During this period, decreased feed intake due to decreased rumen capacity, increased energy and nutrients needs for colostrum and milk synthesis in late pregnancy cause negative energy balance (NEB) and micronutritient deficiencies in dairy cattle [1-3]. Several metabolic (hypocalcaemia, displacement of the abomasum, fatty liver syndrome, and ketosis etc.), infectious and reproductive disorders such as mastitis, dystocia, retained placenta and metritis reported during the early lactation period in high producing herds causes significant economic losses [1,4].

In recent years, the use of glucogenic substances has been increasing in high-producing dairy cows to prevent hepatic lipidosis and ketosis and reduce energy and glucose deficit in the transition period [5]. It has been reported that the use of additives such as glycogen, oils, glycerol, propylene glycol (PG), propionates, monensin, methionine, lysine, choline, niacin, biotin, sodium borate and conjugated linoleic acid (CLA) in the peripartum period may be beneficial in overcoming the periparturient period without any problems [6-8].

Xylitol is an antiketotic, 5-carbon sugar alcohol that stimulates insulin secretion and is used to treat diabetes in humans and cattle with ketosis in Japan. Xylitol can be metabolized as it enters the pentose phosphate cycle even without insulin [9-11]. Sakai et al.[11] determined that blood glucose and insulin response were more effective than glucose, when xylitol treated cattle with ketosis, intravenously. They also reported that xylitol application is beneficial in the treatment of ketosis due to the disappearance of urinary ketone bodies, increased feed consumption and clinical improvement. Toyoda et al.[4] administered xylitol intravenously to healthy and ketotic cattle and determined that it caused a gradual increase in insulin level in ketotic cattle, while a temporary increase in insulin secretion occurred in healthy cattle. Hamada et al.[12] reported that blood glucose levels increased, and ketone bodies decreased when they administered 100 g of xylitol intravenously in cattle with ketosis. One study examined the fattening performance in calves with oral use of xylitol, although there are reports of intravenous use of xylitol in dairy cattle. Although there is no difference in feed consumption in calves given xylitol, glucose and polylol, it has been reported that xylitol is very effective in weight gain [13].

The hypothesis of this study is not the adverse effects on the *in vitro* rumen fermentation of different doses of xylitol, which is an alternative to energy sources in the diet, such as propylene glycol, in the dry period and fresh period (transition period) of dairy cattle. The aim of the present study was determined to be the effect of different doses of xylitol added to the diet on the *in vitro* rumen fermentation, total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen parameters and the total number of protozoa.

Material and Methods

Sample Features

Xylitol (Ksilitol, Nustil®, İstanbul), which is a natural source of birch and beech bark, was supplemented at different rates (0, 2, 4, and 8% dry matter basis) to dairy cattle TMR. Xylitol ratios were determined by modifying the method of Lister and Smithard [14].

Composition of the Dairy Cattle Total Mix Ration

The dairy cattle total mix ration (TMR), which was used in the *in vitro* gas production technique, was composed of 25% corn silage, 13% wheat straw, 20% dried alfalfa herbage [with 17-19% crude protein (CP), 40-44% neutral detergent fiber (NDF)], 16% barley, 8% sunflower meal (28% CP), 9% cottonseed meal (28% CP), 8% wheat bran and 0.1% magnesium sulfate (for anionic diet). The crude protein (CP), net energy lactation (NEL), neutral detergent fiber (NDF) and non-fiber carbohydrate (NFC) content of the dairy cattle ration were 14.2% DM,1.34 Mcal/kg DM, 45.6% and 32.4%, respectively. These rates indicate the dry matter percentages of the feedstuffs.

The Determination of In Vitro Ruminal Digestion Potential and Rumen Fluid Collection

The dairy cattle TMR, which included corn silage, wheat straw, alfalfa herbage, crushed barley grain, sunflower meal, cotton seed meal and wheat bran were used as control. The dairy cattle TMR (control) was prepared in a composition to meet the energy and nutrient requirements of the *Holstein* cattle in the last two months of pregnancy (dry period) [15].

The Determination of In Vitro Total Gas Production, Methane Production, and Estimated Digestion Values

The *in vitro* cumulative total gas production was recorded at 24 h. After 24 h of incubation, the total gas volume was recorded from the calibrated scale in the *in vitro* glass fermenter [16]. After reading the total gas volume, the methane volume in total gas was determined with the infrared methane analyzer (Sensor, Europe GmbH, Erkrath, Germany). The metabolizable energy (ME) and organic matter digestibility (OMd) contents of the dairy cattle TMRs with 0%, 2%, 4%, and 8% Xylitol supplementation as dry matter (DM) were calculated using the equations by Menke and Steingass [17] and Blümmel et al.[18] as follows:

(ME (MJ /kg DM) = 2.20 + 0.136 × Gas24h + 0.057 × CP), (OMd (g/kg DM) = 14.88 + 0.889 × Gas24h + 0.45 × CP + 0.0651 × A). Gas24h = 24 h net gas production (mL/200 mg), CP = Crude protein (g/kg DM), CA = Crude ash content (g/kg DM), EE = Ether extract (g/kg DM).

Determination of Total Ciliate Protozoa Number

At the end of the incubation period, the content of *in vitro* fermentation fluid in glass syringes was used for counting total protozoa [19].

Determination of pH and Ammonia Concentration

The pH value of the filtered *in vitro* fermentation fluid was determined using a digital pH meter (Mettler Toledo S220, Switzerland). The ammonia concentration (mg/L) in the *in vitro* fermentation fluid was determined using a commercial ammonia assay procedure (Megazyme, K-AMIAR 02/20, Wicklow, Ireland) [20].

Determination of Volatile Fatty Acids in the In Vitro Fermentation Fluid

The total gas volume at 24 h of *in vitro* incubation was recorded, and 10 mL of the ruminal fermentation fluid in the glass fermenter was collected into Falcon tubes. The volatile fatty acid (/organic acid) (acetic-AA, propionic-PA, butyric-BA, iso-valeric-IVA, iso-butyric- IBA, valeric-VA and hexanoic - HA acids) molarities in the *in vitro* ruminal fermentation fluid were determined using the gas chromatography (GC) device (Thermo Trace 1300, Thermo Scientific, Waltham, MA, USA). The GC device was equipped with a flame ionisation detector (FID) and a polyethylene glycol column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) (TG-WAXMS, Thermo Scientific, Waltham, MA, USA). The device was operated according to the procedure described by Ersahince and Kara [21]. The percentages of induvial volatile fatty acids in total volatile fatty acids (TVFA), whose molarity was determined by the Xcalibur programme (Thermo Scientific, Waltham, MA, USA), were calculated.

Statistical Analyses

The obtained data were statistically analysed using SPSS 17.0 software (IBM Corp., Armonk, USA). One-way analysis of variance was conducted for variables tested in different doses ofxylitol supplementation. Data were analyzed using the following statistical model:

$$Yij = \mu ij + Si + ei$$

where: Yij = the general mean for each parameter investigated; µ = the mean of xylitol supplementation for each tested parameter; Si = the i th effect of xylitol supplementation on the observed parameters; ei = the standard error The significance of differences in means was revealed using Tukey's multiple range test at $P<0.05$.

Results

The xylitol supplementations at 2% and 4% to dry matter (DM) of dry period TMR of dairy cattle did not change *in vitro* total gas production, *in vitro* methane production, ME and OMd values according to those of control TMR ($P>0.05$). However, 8% xylitol supplementation led to decrease *in vitro* total gas production, *in vitro* methane production, ME and OMd ($P<0.05$) *(Table 1)*.

The molarities of TVFA and percentages of AA, PA, and BA of the *in vitro* rumen fluid of xylitol supplementations at 2%, 4%, and 8% to dairy cattle dry period TMR were like those of control TMR ($P>0.05$). The percentages of IVA, IBA, and VA of *in vitro* rumen fluid decreased with xylitol supplementation, especially 8% xylitol supplementation ($P<0.05$) *(Table 2)*.

The ammonia-nitrogen concentration and number of total ciliate protozoa of *in vitro* fermentation fluid did not change with xylitol supplementation to dairy cattle TMR ($P>0.05$). Besides, 2% and 4% xylitol supplementation to dairy cattle TMR numerically increased the concentration of ammonia-nitrogen and the number of total ciliate protozoa of *in vitro* fermentation fluid ($P>0.05$) *(Fig. 1, Fig. 2)*.

Discussion

Xylitol is a sugar alcohol used as a sweetener for diabetics and also for other purposes (e.g., in chewing gum). Xylitol has attracted global demand mainly due to its insulin-independent metabolism, anti-carcinogenicity, sweetening

Table 1. *In vitro total gas production, methane production and estimated digestion values of dairy cattle TMR with Xylitol supplementation*

Parameters	Xylitol Supplementation to Dairy Cattle TMR*				SEM	P Value	
	0%	2%	4%	8%		L	Q
In vitro total gas production	41.77^{a}	41.63^{a}	36.33^{ab}	29.80^{b}	1.78	0.004	0.179
In vitro methane production	11.57^{a}	12.01^{a}	9.64^{ab}	8.50^{b}	0.52	0.007	0.266
In vitro ME	8.68^{a}	8.66^{a}	7.94^{ab}	7.05^{b}	0.24	0.004	0.181
In vitro OMd	58.83^{a}	58.70^{a}	53.99^{ab}	48.19^{b}	1.58	0.004	0.184

** Xylitol supplementation to DM of dairy cattle TMR, total gas production is as mL/0.2 g DM, Methane production is as mL/0.2 g DM;* ***TMR:*** *total mix ration;* ***OMd:*** *Organic matter digestibility;* ***ME:*** *Metabolizable energy;* ***SEM:*** *Standard error of means*

Table 2. *Ruminal volatile fatty acids of in vitro fermentation for dairy cattle TMR with xylitol supplementation*

Parameters	Xylitol Supplementation to Dairy Cattle TMR*				SEM	P Value	
	0%	2%	4%	8%		L	Q
TVFA, mmol/L	99.86	96.78	94.63	92.69	1.36	0.162	0.385
% values of volatile fatty acids in TVFA							
Acetic acid	70.29	70.22	70.82	70.66	0.11	0.065	0.586
Propionic acid	13.56	13.65	13.43	13.87	0.07	0.782	0.057
Butyric acid	12.21	12.57	12.32	12.27	0.06	0.933	0.181
Iso-valeric acid	1.60^{a}	1.37^{ab}	1.27^{b}	1.18^{b}	0.05	0.002	0.024
Iso-butyric acid	0.91^{a}	0.81^{b}	0.77^{b}	0.73^{b}	0.02	0.002	0.007
Valeric acid	1.12^{a}	1.03^{ab}	1.03^{ab}	0.95^{b}	0.02	0.025	0.018
Hexanoic acid	0.31	0.33	0.34	0.31	0.005	0.052	0.682

** Xylitol supplementation to DM of dairy cattle TMR,* ***TVFA:*** *total volatile fatty acids are sums of acetic acid + propionic acid + butyric acid + iso-valeric acid + iso-butyric acid + valeric acid + hexanoic acid;* ***TMR:*** *Total mix ration;* ***SEM:*** *Standard error of means*

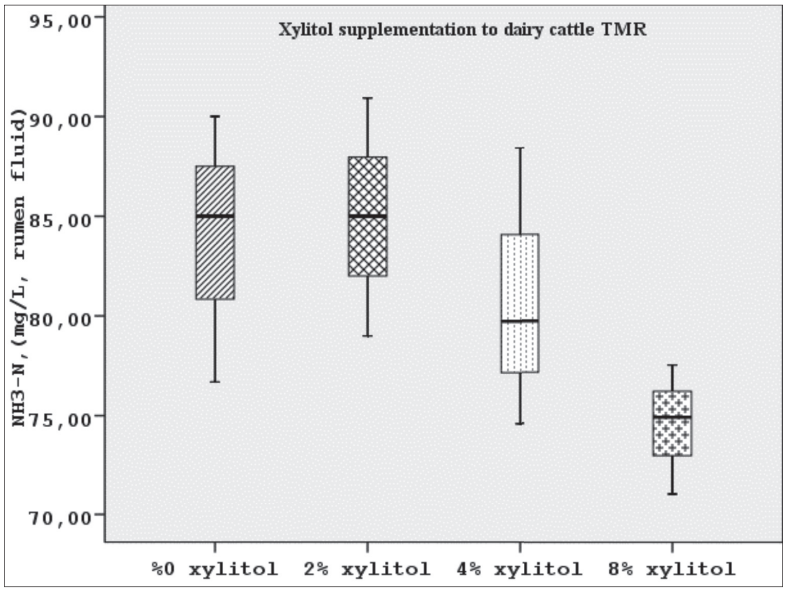
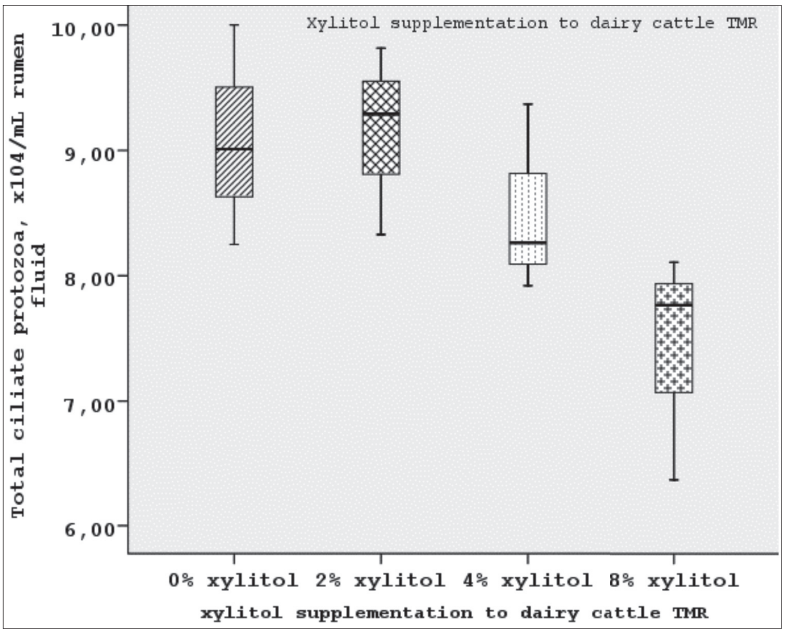


Fig 1. Ammonia-nitrogen concentration of ruminal fluid the *in vitro* fermentation for dairy cattle TMR with Xylitol supplementation (P value; Linear: 0.529 and Quadratic: 0.147)

Fig 2. Total ciliate protozoa number of ruminal fluid the *in vitro* fermentation for dairy cattle TMR with xylitol supplementation (P values; Linear: 0.815 and Quadratic: 0.188)



power similar to sucrose, and pharmacological properties [22]. However, there is not enough information about the efficacy of xylitol in rumen fermentation in the literature and the dose to be added to the feed. In the present study, the xylitol supplementations at 2% and 4% to DM of dry period TMR of dairy cattle did not change *in vitro* total gas production, *in vitro* methane production, ME and OMd values according to those of control TMR demonstrated that did not change normal ruminal fermentation of these doses of xylitol. However, in the presented study, it was understood that the addition of 8% xylitol to the TMR can reduce *in vitro* ruminal fermentation values. The results of the study show that the high use of this prebiotic sugar alcohol (xylitol) in the rumen may have negative effect. The fact that there is no study on the effect of the use of xylitol in the diet on the fermentation values in ruminants causes the study results not to be discussed with ruminants. Xu et al.[23] stated that xylitol significantly enhanced the level of butyrate synthesizing bacteria such as *Clostridium* and *Phascolarcto bacterium* in the *in vitro* colonic simulation of human. The same researchers showed that xylitol increased the production of propionic acid and butyrate in the *in vitro* colonic simulation of humans [23].

It is vital to reduce the methane emissions of ruminants (especially in dairy cattle enterprises) due to their undesirable contribution to global warming. In recent years, there have been studies on this subject with different additives or alternative feedstuffs materials. The aim here is to reduce methane production without adversely affecting rumen fermentation and digestion in the rumen (without adversely affecting the utilization level of energy and nutrients in the feed) [24-26]. In the presented study, despite reducing methane production with the addition of 8% xylitol, it is not desired that the *in vitro* total gas production and ME and OMd values be also reduced. In this respect, using of xylitol as an anti-methanogenic additive is not recommended at these doses.

In the study, TVFA, AA, PA, BA, IBA, VA and IVA molarities of the *in vitro* rumen fluid were compatible with reference values [21,27]. The molarities of TVFA, percentages of AA, PA and BA of the *in vitro* rumen fluid of xylitol supplementations at 2, 4 and 8% to DM of dry period TMR of dairy cattle were like those of control TMR. In another study, the molarity of BA increased with increasing xylitol supplementation doses of the *in vitro* ruminal batch culture (at 0.85, 2.13, and 4.25 g/L concentrations) for 12 h [28]. Researchers demonstrated that these two lower xylitol concentrations decreased the percentage of PA in TVFA [28]. Using a ration consisting of roughage, the researchers differed from our study results [28]. The total mix ration of dairy cattle was used in the current study, and it was rich in soluble and digestible carbohydrates. In rumen fermentation, soluble and easy fermentable carbohydrates (pentoses; L-arabinose, D-ribose, and D-xylose, pentitols; L-arabinitol, ribitol, and xylitol) have fermented as firstly and, they have demonstrated high gas production and fermentation kinetics. Then the digested carbohydrates (such as starch) have fermented, followed by structural carbohydrates such as cellulose and hemicellulose [15]. The linearly decreasing of IVA, IBA and VA percentages in the TVFA of *in vitro* rumen fermentation fluid in the present study can show the potential of this polyol compound to alter rumen fermentation in a dose-dependent manner.

Some of the nitrogenous compounds (true proteins and other nitrogen-containing compounds) in the rumen are decomposed into ammonia in the rumen. Some of this ammonia (in the presence of sufficient alpha keto-acids) is absorbed into the microbial protein. A part of it is absorbed from the rumen wall and comes back to the rumen with saliva [15,29]. However, proteinaceous compounds passing through the rumen and microbial proteins produced in the rumen can be taken from the intestines as amino acids. In the presented study, the fact that both ruminal ammonia nitrogen and ruminal protozoa levels did not change shows that these levels of xylitol used in TMR do not have a negative effect on protein metabolism and milk yield.

In conclusion, it was thought that the use of xylitol compound as an energy source in dairy cattle rations did not have a negative effect on ruminal fermentation at 2% and 4% doses and these doses should be applied in the *in vivo* feeding trials.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

Funding Support

There is no funding source.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The rumen contents used in this study were obtained from the slaughterhouse. Ethics committee approval is not required for this study because of performing *in vitro* in the laboratory.

Author Contributions

YS and ÖA: the hypothesis of this study; ÖA and KK: work management, article writing; KK, SEÖ, SY and MAÖ: experimental procedure follow-up; YS, ÖA, KK, SEÖ, SY and MAÖ: literature review, review of results, final decision.

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Research Article

Phytochemical Analysis and Antimicrobial Effect of Essential Oil and Extract of *Loranthus europaeus* Jacq. on *Acinetobacter baumannii, Staphylococcus aureus,* and *Pseudomonas aeruginosa*

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Abstract: With the increase in the use of antibiotics and resistance against them, attention has been paid to natural remedies with the possibility of lower resistance and side effects. In this study, the essential oil of *Loranthus europaeus* Jacq. was identified chemically and the antimicrobial effects of the essential oil and extract on bacterial agents such as *Acinetobacter baumannii, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were investigated. *L. europaeus* leaves were collected from the Ilam mountains and after drying, essential oil and hydroalcoholic extract were prepared. The chemical compositions of the essential oil and extract of *L. europaeus* plant were measured by Headspace-solid phase microextraction (HS-SPME), Gas chromatography-mass spectrometry (GC-MS), and High Performance Liquid Chromatography (HPLC) methods. The results showed that the main component of *L. europaeus* extract was Rutin (223 µg/mL). The bacterial strains were isolated from clinical samples, and the microbroth dilution method by Clinical and Laboratory Standards Institute (CLSI) method were used to evaluate the Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC for the *L. europaeus* leaf extract were 6 µg/mL and 196 µg/mL on *S. aureus,* respectively. It had no significant effect on the strains of *A. baumannii* and *P. aeruginosa. L. europaeus* leaf essential oil also had no antimicrobial effect on bacterial sauces. The results showed that the plant extract of *L. europaeus* might be used to treat infections due to its very low MIC against *S. aureus.*

Keywords: *Loranthus europeaus Jacq., Antibactrial, Phytochemistry, GC-SPME, HPLC, Rutin*

Loranthus europaeus Jacq. Uçucu Yağ ve Özütünün Fitokimyasal Analizi ve *Acinetobacter baumannii, Staphylococcus aureus* ve *Pseudomonas aeruginosa* Üzerine Antimikrobiyal Etkisi

Öz: Antibiyotik kullanımının ve bunlara karşı direncin artmasıyla birlikte daha düşük direnç ve yan etki olasılığı olan doğal ilaçlara dikkat çekilmeye başlanmıştır. Bu çalışmada, *Loranthus europaeus* Jacq. uçucu yağı kimyasal olarak tanımlandı ve uçucu yağ ve ekstraktın *Acinetobacter baumannii, Staphylococcus aureus* ve *Pseudomonas aeruginosa* gibi bakteriyel etkenler üzerine antimikrobiyal etkileri araştırıldı. *L. europaeus* yaprakları Ilam dağlarından toplandı ve kurutulduktan sonra uçucu yağ ve hidroalkolik ekstrakt hazırlandı. *L. europaeus* bitkisinin uçucu yağı ve ekstraktının kimyasal bileşimleri, Headspace-katı faz mikroekstraksiyon (HS-SPME), Gaz kromatografisi-kütle spektrometrisi (GC-MS) ve Yüksek Performanslı Sıvı Kromatografisi (HPLC) yöntemleri ile ölçüldü. Sonuçlar, *L. europaeus* özütünün ana bileşeninin Rutin (223 µg/mL) olduğunu gösterdi. Bakteri suşları klinik örneklerden izole edildi ve minimum inhibitör konsantrasyonu (MIC) ve minimum bakterisidal konsantrasyonu (MBC) değerlendirmek için Klinik ve Laboratuvar Standartları Enstitüsü (CLSI) yöntemine göre mikro broth seyreltme yöntemi kullanıldı. *L. europaeus* yaprak özütünün MIC ve MBC değerleri, *S. aureus* için sırasıyla 6 µg/mL ve 196 µg/mL saptandı. *A. baumannii* ve *P. aeruginosa* suşları üzerine önemli bir etkisi olmadı. *L. europaeus* yaprağı esansiyel yağı, bakteri sosları üzerine antimikrobiyal etkiye sahip değildi. Sonuçlar, *L. europaeus* bitki özütünün, *S. aureus*'a karşı çok düşük MIC'i ile enfeksiyonları tedavi etmek için kullanılabileceğini gösterdi.

Anahtar sözcükler: *Loranthus europeaus Jacq., Antibakteriyel, Fitokimya, GC-SPME, HPLC, Rutin*

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Introduction

Loranthus europaeus Jacq. is a parasitic plant of the *Loranthaceae* family belonging to the genus *Loranthus* [1]. *Loranthus* is currently a major pest infecting a large part of oak trees [2]. This plant clings to the trunks of the host trees for germination, establishment and absorbing water and materials from it [3]. It is not harmful in its natural state, but it can damage trees and shrubs [4,5]. *Loranthus micranthus* is used in traditional medicine to treat diabetes, schizophrenia, blood clots, and as an immune system stimolator [6]. It is also used to treat infertility, epilepsy, cardiovascular disease, menopausal syndrome, rheumatoid arthritis, and agglutination [7]. It has been shown that there are components such as quercetin, rutin, and epicatechin in this plant [2].

Infectious diseases are widespread diseases in the world that impose great costs on human societies. The diseases death rate is increasing in the world day by day. In recent decades, synthetic antibiotics have played an important role in the treatment of infectious diseases [8]. Today, with the increasing use of antibiotics, the resistance of pathogenic microbial species is increasing and spreading. Resistant pathogenic bacteria have made it difficult to treat infectious diseases. So, researches to discover antibiotic compounds from new sources have been increased [9,10]. Therefore, researchers are searching in nature to discover compounds that have therapeutic potential for use in infectious diseases [11]. The antimicrobial properties of medicinal plants have been considered and used in the treatment of infectious diseases [12].

In this study, the essential oil of *L. europaeus* was identified and the antimicrobial effects of the essential oil and extract on bacterial infectious such as *Acinetobacter baumannii*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* were investigated.

S. aureus stays as our natural flora, and yet sometimes threatens our life as a tenacious pathogen. In addition to its ability to outwit our immune system, its multi-drug resistance phenotype makes it one of the most pathogenic bacteria in the history of antibiotic therapy. Therefore, the presence of natural and herbal compounds that have strong antibacterial properties and are not harmful to human health can be very useful in the treatment of nosocomial infections. The aim of this study was to chemically analyze *S. aureus* plant and determine the active ingredients of this plant and to investigate the antibacterial properties of this plant.

Material and Methods

This study was conducted with the approval of Ilam University of Medical Sciences with the ethics code IR.MEDILAM.REC.1400.074.

Plant Collection

This experimental study was performed in Biotechnology and Medicinal Plants Research Center, Ilam University of Medical Sciences, Ilam, Iran (June 2020 to November 2020). The leaves of the Laurentus plant were collected in June 2020 from the Qalandar region of Ilam city (Ilam province, western Iran) and dried in the shade at room temperature. The identity of this plant was authenticated by the voucher specimens (NO 623) deposited in the Department of Horticulture, Faculty of Agriculture, Ilam University.

Preparation of Essential Oil and Phytochemical Analysis of the Plant

Plant essential oil was extracted by the HS-SPME (Head-space-solid Phase Microextraction) method and the chemical composition of plant leaves was identified by the GC-MS (Gas Chromatography-Mass Spectrometry) method [13]. Extraction of chemical compounds of *L. europaeus* plant was done by HS-SPME method. In this study, the essential oil of *L. europaeus* seed was extracted by HS-SPME technique. In the HS-SPME technique, about 2 grams of dried plant powder was placed in a vial and the vial temperature rised between 60 and 70°C (Extraction time: 20 min). The temperature condition was in the optimal state, causing the extraction of vapors of substances in plant essential oil in the space above the solid surface in a saturated form. The SPME syringe was then placed in the upper space of the container with the lid closed and the material in the plant vapors was absorbed by the silica phase in the needle of the device. After sufficient time and saturation of silica fiber, the volatile compounds of the fiber were placed directly in the input part of the GC-MS device and due to the temperature of the input port, the material in the fiber was desorbed and entered into the GC-MS device and identified.

GC-MS Device Conditions

The device condition was as follows:

The gas chromato-graph (Agilent 6890N) was coupled to the Agilent 5973 bulk detector. Column: HP-5. (30 m length, 0.25 mm (ID), 0.25 µm fixed phase thickness). Type of injection: split/gap and column temperature program: 50°C, holding time 0.00 min; 200°C temperature, holding time, 0.00 min and 5°C/min and 240 min temperature, holding time 0.00 min and 10°C/min. Carrier gas: He (99.999%); Injection type: no gap; Library: Willey 7n; Injector temperature: 250°C and flow rate: 0.9 mL per minute. Extraction mode: (HS-SPME); SMPE fibers: PDMS thickness 100 micrometers (SUPELCO); Sample weight: 0.5 g; Extraction temperature: 60°C; Extraction time: 20 min; Ultrasound time: 10 min (Euronda ultra-sound instrument, Italy) and disposal time in GC-MS injector port: 3 min [14].

Preparation of Hydroalcoholic Extract

The dried Laurentus plant was ground with a mill and poured 30 g of the resulting powder into the cartridge and in Soxhlet using 400 mL of 30% ethanol solution, 40% methanol, and 30% water at 75°C. It was placed for 3 h to complete the extraction. The resulting green hydroalcoholic solution (400 mL) was placed in a rotary apparatus. The solvents in the extract were removed by the rotary device (IKA HB 10, Germany) at 30 rpm and 50°C for one hour to evaporate ethanol, water, and methanol. The resulting *L. europaeus* extract was dried for 3 days. Then the extract was lyophilized and kept stored at -20°C. The amount of extract obtained was 1. 788 g (5.96%).

Preparation and Identification of Active Ingredients

Rutin standards were purchased from Sigma Aldrich (USA) and ethanol and methanol solvents with HPLC (High-performance liquid chromatography) analysis grade were purchased from Merck Company. To identify and determine the exact amount of Rutin in the extracted extract, high-performance liquid chromatography (HPLC) was performed based on a previously reported method. Under similar conditions, a standard solution of methanol-soluble Rutin was also analyzed. The standard Rutin solution was prepared in pure methanol at a concentration of one-tenth of a milligram per milliliter and then diluted in the same solvent to concentrations of 10, 20, 40, 80, 160, and 320 µg/mL. Two samples prepared from rutin extract, diluted 1:50 and 1:100 with methanol, were centrifuged and then filtered to precipitate impurities. All prepared concentrations were injected separately by HPLC. The samples were identified and measured after passing through the C18 column at a wavelength of 254 nm and the chromatogram curve of each was plotted. The curves of the compounds of *Laurentus hydroalcoholic* extract were compared and calculated with the standard Rutin [15].

HPLC Conditions

Analyzes were performed with the HPLC device of Knauer company (Germany) (PLATIN BLUE model). Finally, the best conditions were: Column type: HPLC Column, 250 x 4.6 mm, Eurospher II, 100-5 C18. Mobile phase: formic acid solution with a concentration of 0.2% and acetonitrile 70% and 30%, respectively. Moving phase flow rate: 1 mL per min; Column chamber temperature: 25°C; Injection loop volume: 50 microliters.

Preparation of Bacterial Strain and Bacterial Stock

S. aureus strain (ATCC 12600) and *P. aeruginosa* (ATCC 27852) were purchased from the Iranian Research Organization for Science and Technology. *A. baumannii* isolates associated with infection in patients admitted to the burn ward of a teaching hospital in Ilam were identified as *A. baumannii* by API 20NE and gyrB multiplex PCR [16].

Antimicrobial Method (Microbroth Dilution)

Antibacterial activities of *Loranthus* essential oil and leaf extract (MIC and MBC) were determined using the micro-broth dilution method. In microbiology, the minimum inhibitory concentration (MIC) was considered the lowest concentration of a chemical, which prevented the visible growth of a bacterium or bacteria. The Minimum Bactericidal Concentration (MBC) was considered the lowest concentration of the antibacterial agent required to kill the bacterium over the fixed. Initially, a stock of the plant extract was prepared. In a 96-well microtiter plate, 50 µL of Muller-Hinton broth culture was added to each well. Then, 50 µL of prepared stocks (essential oil/extract) was added to rows 1 and 2, and the dilution was performed from the second to the tenth row. Finally, 50 µL of *A. pneumoniae*, *S. aureus*, and *P. aeruginosa* (24-h culture), equivalent to half-MacFarland 5×10^5 CFU/mL, was added to wells two to ten. gentamicin, cholestin and methicillin, and dimethylsulfoxide (DMSO) was used as a positive and negative control, respectively. Plates were incubated at 37°C for 24 h. After incubation, 30 µL of 2, 3, and 5-trinyl tetrazolium chloride was used as bacterial growth visual index [17]. The antimicrobial test was repeated three times for each group. The proposed CLSI protocol (2014) was also used to determine the MBC [17]. Standard antibiotics methicillin, cholestin, and gentamicin were used as controls [18].

Results

In this study, the essential oils and volatiles of Lorenthus leaves were extracted by solid-phase microextraction in the upper space (HS-SPME) and the chemical compounds were phytochemically analyzed by GC-MS technique. The results of the phytochemical analysis showed that the lorenthus leaf had 35 chemical compounds. According to the results, the most essential compounds of Laurentus leaf essential oil included phytol (18.91%), dodecane (10.30%), trans-Caryophyllene (5.86%), 3-Hexen-1-ol, benzoate, (Z) (5.21%), Neryl acetone (4.78%), and beta lonone (4.53%), respectively. The results of the percentage of other chemical compounds are detailed in *Table 1* and *Table 2*. Also, the chromatogram of *Laurentus* essential oil is shown in *Fig. 1*.

The HPLC results are shown in *Fig. 2*. Accordingly, the main composition of *Laurentus* hydroalcoholic extract was Rutin (223 µg/mL) based on HPLC. HPLC-PDA chromatograms and UV spectra of Rutin are shown in *Fig. 3*.

The microbroth dilution method was used to determine the MIC and MBC of lorenthus extract and essential oil. According to the results, the MIC and MBC for the hydro-alcoholic extract of lorenthus leaf were 6 µg/mL and 196 µg/mL *S. aureus*, but this extract did not affect

Table 1. *Chemical compounds of Laurentus leaf essential oil*

Compound	Retention Time	Percent	Molecular Formula	Compound	Retention Time	Percent	Molecular Formula
Dodecanal	12.018	2.02	$C_{12}H_{24}O$	Neryl acetone	23.42	4.78	$C_{13}H_{22}O$
2-Octanone	12.252	0.30	$C_8H_{16}O$	Heneicosane	23.557	1.46	$C_{21}H_{44}$
Menthone	13.761	1.47	$C_{10}H_{18}O$	Beta.-Ionone	24.403	4.53	$C_{13}H_{20}O$
Menthol	14.539	1.86	$C_{10}H_{20}O$	Pentadecane	24.62	2.23	$C_{15}H_{32}$
Dodecane	15.19	10.30	$C_{12}H_{26}$	Hexadecane	25.369	1.66	$C_{16}H_{34}$
Decanal	15.487	1.46	$C_{10}H_{20}O$	Hexatriacontane	25.838	3.52	$C_{36}H_{74}$
Fenchyl acetate	15.956	0.73	$C_{12}H_{20}O_2$	Decane, 5,6-dimethyl-	25.986	3.23	$C_{12}H_{26}$
Isobornyl acetate	18.191	0.58	$C_{12}H_{20}O_2$	Tetratetracontane	26.278	0.67	$C_{44}H_{90}$
Menthyl acetate	18.465	2.61	$C_{12}H_{22}O_2$	3-Hexen-1-ol, benzoate, (Z)-	26.666	5.21	$C_{13}H_{16}O_2$
Tridecane	18.562	1.91	$C_{13}H_{28}$	Farnesol	27.004	1.09	$C_{15}H_{26}O$
Tridecane, 5-methyl-	20.311	2.97	$C_{14}H_{30}$	Phytol	27.198	18.91	$C_{20}H_{40}O$
2-Methyltetradecane	20.848	2.06	$C_{15}H_{32}$	Dotriacontane	28.364	1.71	$C_{32}H_{66}$
Copaene	21.111	1.16	$C_{15}H_{24}$	Heptadecane	29.564	1.11	$C_{17}H_{36}$
2-Tetradecene, (E)-	21.665	3.79	$C_{14}H_{28}$	1-Eicosanol	29.656	3.71	$C_{20}H_{42}O$
Tetradecane	21.768	0.37	$C_{14}H_{30}$	Heneicosane	31.37	1.29	$C_{21}H_{44}$
Trans-Caryophyllene	22.466	5.86	$C_{15}H_{24}$	Cyclopentadecane	31.97	1.09	$C_{15}H_{30}$
Camphene	22.746	0.46	$C_{10}H_{16}$	Hexadecanoic acid	33.182	3.41	$C_{16}H_{30}D_2O_2$
Alloaromadendrene	23.037	0.48	$C_{15}H_{24}$				

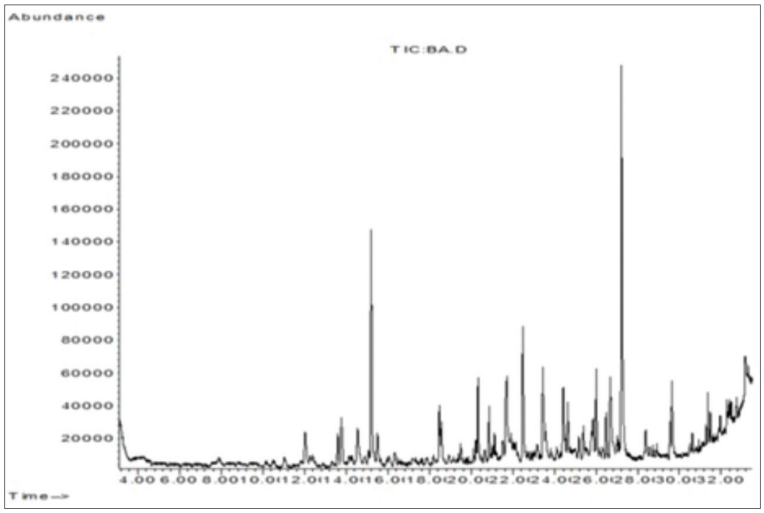

Fig 1. Gas chromatography-mass spectrometry analysis in the essential oil of *Loranthus europaeus*

Table 2. *Active substance, chemical formula and molecular formula of the main components of Laurentus plant*

Active Substance	Chemical Formula
Phytol	$C_{20}H_{40}O$
Dodecane	$C_{12}H_{26}$
Trans-Caryophyllene	$C_{15}H_{24}$
3-Hexen-1-ol, benzoate, (Z)	$C_{13}H_{16}O$
Neryl acetone	$C_{13}H_{22}O$
Beta-lonone	$C_{13}H_{20}O$

the strains of *A. baumannii* and *P. aeruginosa*. Lorenthus leaf essential oil also had no antimicrobial effect on any of the bacterial sauces in this study. The results of the antimicrobial effect of lorenthus essential oil and leaf extract are shown in *Table 3*.

Discussion

Today, the use of medicinal plants in the treatment of various diseases has been considered by researchers. In this regard, some medicinal plants components are used for a variety of disorders and diseases [19]. Phenolic compounds as well as flavonoids, because they are important bioactive compounds for antioxidant effects, have always been considered for the prevention of disease and human health [20].

It has been documented that the fruit extracts of *L. europaeus* (with MIC=0.16±0.04 and 0.28±0.05 $mg.mL^{-1}$ and MBC=0.20±0.01 and 0.38±0.02 $mg.mL^{-1}$) have been effective on *S. aureus* MRSA and *Listeria monocytogenes* species, respectively [21]. The antimicrobial effects of volatile oils and extracts of lorenthus fruit may act as immuomoduialtors during bacterial infection containing substances that act as chemotactic agents for neutrophils and promote macrophage activity [22].

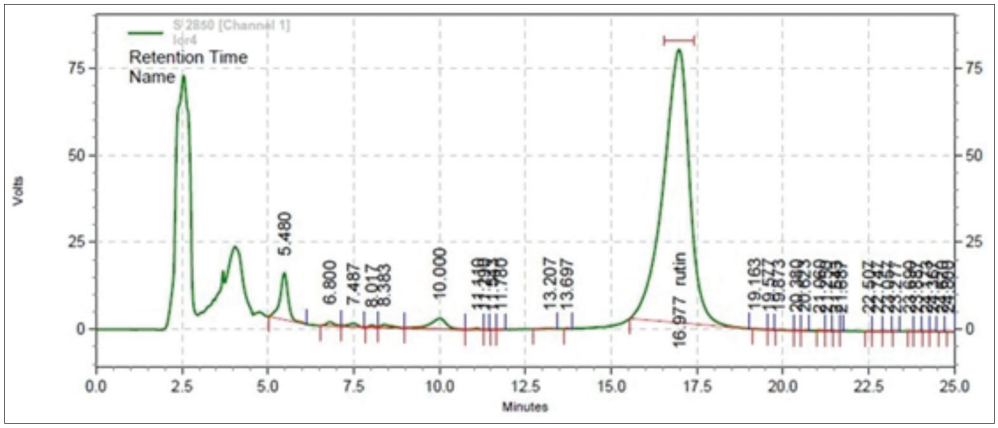

Fig 2. HPLC chromatogram of phenolic compounds present in *Loranthus europaeus* leaves extracts and Rutin

Fig 3. HPLC-PDA chromatograms and UV spectra of Rutin

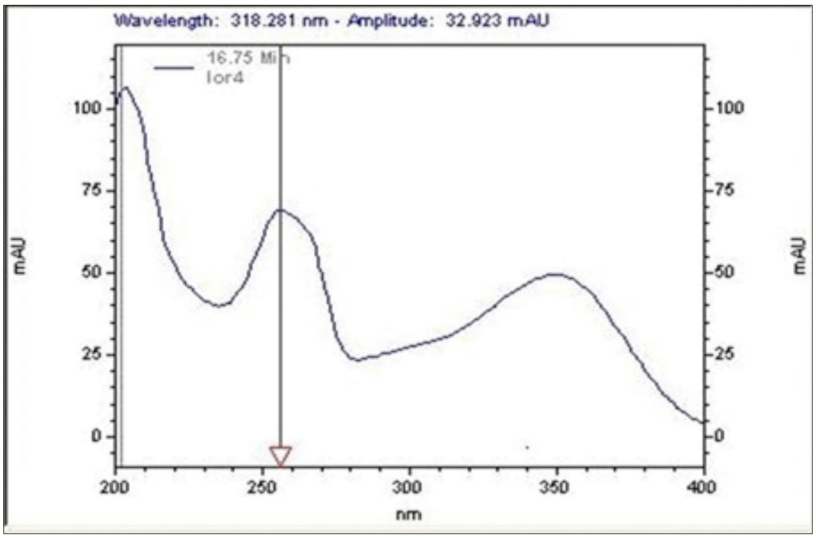

Table 3. *Results of MIC and MBC of Laurentus essential oil and hydroalcoholic extract*

Bacterial Strain	Antibiotics						Essence		Hydroalcoholic Extract	
	Gentamicin		Colestin		Methicillin					
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
Acinetobacter baumannii	IF	IF	75	25	IF	IF	IF	IF	IF	IF
Staphylococcus aureus	IF	IF	IF	IF	384	384	IF	i.f.	196	6
Pseudomonas aeruginosa	12.35	12.35	IF	IF	IF	IF	IF	IF	IF	IF

IF = Ineffective

It has been shown that the alcoholic extract of *L. europaeus* has an inhibitory effect on the methicillin-resistant *S. aureus*, which was 17.28 mm at the concentration of 20 mg/mL, followed by the concentrations of 100, 50, 25 mg/mL which the diameter of inhibition zones were 13.28, 10.57, 8.42 mm, respectively [18]. A previous study showed that lorenthus could affect methicillin-resistant *S. aureus* [23]. In our study, *Loranthus* extract was effective against infection *S. aureus*. Also, our results showed that tannins and flavonoides existed in *L. europaeus*, deposited in the bacterial cell membrane, and inhibited the action of metabolic enzymes leading to bacterial death.

Based on the results obtained from the analysis of plant essential oil, the most compounds in *L. europaeus* had plant essential oil included hexadecanoic acid and 1-eicosanol. It has been shown that the essential oils of *Solanum sisymbriifolium* containing hexadecanoic acid and 1-eicosanol showed antibacterial activity against *S. aureus* in 60 and 80 µg/mL for fruit and flower, respectively [24]. Today, the importance of effective compounds of medicinal plants has become especially important in pharmaceutical science [25,26]. Medicinal plants have different compounds that can be used in various industries such as health, medicine and pharmaceutical industry [27,28]. Many herbal

remedies are derived from compounds found in herbs [29,30]. Rutin ($C_{27}H_{30}O_{16}$) or Phytomelin is a flavonol glycoside found in many plants including buckwheat, lorenthus, and eucalyptus acts as a metabolite and an antioxidant. It is a disaccharide derivative, a quercetin glucoside, a tetra hydroxyl flavone, and a rutinoside [31]. Rutin has many therapeutic effects on blood pressure, diabetes, cancer, sedatives, analgesics, blood cholesterol, cataract [32]. Other Rutin effects include antibacterial, antimalarial, antiviral, and antifungal activities [33]. It has been studied for antimicrobial activity against different strains of bacteria. Also, it has antimicrobial activity against *Escherichia coli*, *Proteus vulgaris*, *Shigella sonei*, *Klebsiella,* and *Bacillus subtilis* [25,34-36]. The main reason for the lorenthus hydroalcoholic extract effectiveness might be the high concentration of Rutin. Extraction of herbal active ingredients and their implications in the treatment of diseases are expanding [35,36]. The results of extract analysis showed that the main ingredient of this plant is Rutin. The very low MIC extract of the *L. europaeus* has the potential to be used as alternative medicine in the treatment of *S. aureus* infections. Its toxicological studies on human cells should be considered.

Availability of Data and Materials

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

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Competing of Interest

The authors declared no competing interests.

Authors' Contribution

NA, AA and EK conceived and supervised the study. MB, VHK and MP collected and analyzed data. VHK made antimicrobial and MB and AA made phytochemical measurements. The first draft of the manuscript was written by MB and NA and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Hydrogen Relieves Neuropathic Pain in Diabetic Rats by Inhibiting MCP1 and CCR2 Expressions

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Abstract: We aimed to explore the mechanism for hydrogen in the treatment of neuropathic pain in diabetic rats. Eight-week-old male SD rats were randomly divided into control, model and hydrogen treatment groups. The hydrogen treatment group was intraperitoneally injected with hydrogen-rich saline daily in 7th and 8th weeks after modeling. Before and 2, 4, 6 and 8 weeks after modeling, the neurological function, behavioral changes and levels of inflammatory factors TNF-α and IL-6 in the sciatic nerve were detected, and MCP1 and CCR2 protein expressions were measured by Western blotting. Compared with the model group, the hydrogen treatment group had significantly increased motor nerve conduction velocity, thermal withdrawal latency and mechanical withdrawal threshold ($P<0.05$). The significantly higher levels of TNF-α and IL-6 in the sciatic nerve of the model group than those of the control group decreased in the hydrogen treatment group ($P<0.05$). The protein expressions of MCP1 and CCR2 in the sciatic nerve of the model group significantly exceeded those of the control group. Such expressions of the hydrogen treatment group were lower than those of the model group. Hydrogen alleviated the inflammatory response of peripheral nerves in diabetic rats by inhibiting the MCP1-CCR2 signaling pathway, thus mitigating neuropathic pain.

Keywords: *Diabetic neuropathy, Hydrogen, MCP1, CCR2, TNF-α, IL-6*

Hidrojen, MCP1 ve CCR2 Ekspresyonlarını Engelleyerek Diyabetik Sıçanlarda Nöropatik Ağrıyı Hafifletir

Öz: Diyabetik sıçanlarda nöropatik ağrının tedavisinde hidrojen mekanizmasını araştırmayı amaçladık. Sekiz haftalık erkek SD sıçanları rastgele olarak kontrol, model ve hidrojen sağaltım gruplarına ayrıldı. Hidrojen uygulanan gruba, modellemeden sonra 7. ve 8. haftalarda günlük olarak hidrojence zengin salin intraperitoneal olarak enjekte edildi. Modellemeden önce ve 2, 4, 6 ve 8 hafta sonra, siyatik sinirdeki nörolojik fonksiyonlar, davranış değişiklikleri ve inflamatuar faktörlerden TNF-α ve IL-6'nın seviyeleri tespit edildi. MCP1 ve CCR2 protein ekspresyonları ise Western blot ile ölçüldü. Model grup ile karşılaştırıldığında hidrojen uygulanan grupta, motor sinir iletim hızı, termal geri çekme gecikmesi ve mekanik geri çekme eşiği önemli ölçüde artmıştı ($P<0.05$). Kontrol grubuna göre model grubunun siyatik sinirlerdeki önemli ölçüde yüksek TNF-α ve IL-6 seviyeleri, hidrojen uygulanan gruba göre daha azdı ($P<0.05$). Model grubuna ait siyatik sinirlerdeki MCP1 ve CCR2'nin protein ekspresyonları, kontrol grubuna göre önemli ölçüde artmıştı. Hidrojen uygulanan grubun bu tür protein ekspresyon seviyeleri, model grubuna göre daha düşüktü. Hidrojen, MCP1-CCR2 sinyal yolunu inhibe ederek diyabetik sıçanlarda periferik sinirlerin inflamatuar yanıtını azalttı ve böylece nöropatik ağrıyı hafifletti.

Anahtar sözcükler: *Diyabetik nöropati, Hidrojen, MCP1, CCR2, TNF-α, IL-6*

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Introduction

Diabetes is one of the most influential and harmful diseases in the world, which leads to metabolic damage, cardiovascular disorders and obesity, accompanied by vascular complications. Damage to vascular endothelial cells caused by hyperglycemia results in microvascular complications such as diabetic peripheral neuropathy (DPN), diabetic nephropathy and retinopathy. DPN is the most common yet intractable comp-lication of diabetes, usually manifested as distal symmetrical sensorimotor polyneuropathy and autonomic neuropathy [1]. Over 50% of diabetic patients have peripheral neuropathy as the chronic disease develops, of whom 40-50% suffer from pain symptoms such as hyperalgesia, numbness and paralgesia. Some of them may even suffer from gangrene in the lower limbs with the aggravation of disease and have to receive amputation [2]. Diabetes is the main cause for lower limb amputation. There are approximately 80.000 diabetic patients undergoing amputation annually in the USA only. The pain and risk of amputation caused by diabetes have severely affected the quality of life as well as physical and psychological healths, and hugely burdened the society and families. However, due to the unknown pathogenesis of diabetes, patients' suffering can be relieved only by controlling blood glucose level and alleviating pain symptoms. Hence, it is urgent to find new drugs based on multiple pathogenic mechanisms.

Many factors are involved in the pathogenesis of DPN, including oxidative stress, pre-inflammatory changes and formation of advanced glycation end products. These processes lead to many types of cell damage, such as neuronal damage and vascular endothelium impairment. Among all the factors, oxidative stress and inflammatory mechanism play central roles in the onset of diabetic neuropathy. It is well-documented that monocyte chemoattractant protein-1 (MCP-1) and its specific receptor C-C chemokine receptor 2 (CCR2) play important roles as inflammatory mediators in the pathophysiological processes of many diseases [3-5]. They participate in all stages of inflammatory response, covering adhesion, migration, removal of inflammatory substances and repair, and also essentially regulate inflammation, immunity, infection and other diseases.

Hydrogen is a novel antioxidant found in recent years. In the past, most biologists considered hydrogen physiologically inert. Cytological and molecular biological studies have proven that hydrogen can selectively eliminate hydroxyl radical and peroxynitrite anion [6]. In 2009, Mao et al.[7] dissolved hydrogen into normal saline at high pressure to prepare 0.6 mmol/L hydrogen-rich saline (HRS) or hydrogen-saturated medium for *in vitro* and *in vivo* experiments. Since then, HRS has been widely applied to study different disease models, but the specific mechanism remains largely unknown. Compared with other antioxidants, hydrogen is typified by selectivity, no toxicity, strong permeability, no residue and low cost, with well-established protective and therapeutic effects on 63 disease models. Nevertheless, its influence on DPN has never been reported hitherto. Since DPN is closely related to oxidative stress damage and inflammatory response, we postulated that HRS may be clinically effective for treating DPN.

Material and Methods

Ethical Approval

The study has received ethical approval of Affiliated Jiangning Hospital of Nanjing Medical University, China, (AJHNMU-201903006).

Experimental Animals

SPF 8-week-old healthy male SD rats weighing 200-220 g were purchased from Shanghai SIPPR-Bk Lab Animal Co., Ltd. [China, license: SCXK (Shanghai) 2018-0007]. All rats were fed in a well-ventilated environment with free access to food and water on a 12 h/12 h light/dark cycle.

Establishment of Diabetic Model

After fasting for 12 h, healthy male SD rats were intraperitoneally injected with streptozotocin (STZ) at a concentration of 1% (65 mg/kg) dissolved in sodium citrate buffer (pH 4.2-4.5). In control group, an equal volume of sodium citrate buffer was injected only. All rats had free access to food and water. After 48 h, the blood was drawn via the caudal vein to measure the fasting blood glucose using Accu-Chek blood glucose meter and test paper (Roche, Germany). Fasting blood glucose ≥16.67 mmol/L indicated successful modeling.

Estimation equation for sample size: $n = (t_{0.05} \cdot S_d)^2/d^2$, where S_d is the variance of differences between pairs obtained in the pre-experiment; d is the mean difference when the expected difference is significant; $t_{0.05}$ is the t value when $\alpha=0.05$ at a given df. In this study, t=2 and pre-experiment $S_d=1.2$, so $n= (2\times1.2)^2/1.1^2=5.236$. Finally, 6 rats were used for each group.

Preparation of HRS

Hydrogen was dissolved in normal saline using a hydrogen generator under the pressure of 0.4 MPa till the saturated condition, such a condition was maintained for 6 h, and it was stored in a refrigerator at 4°C prior to use. HRS should be freshly prepared weekly, so that the hydrogen concentration was kept at 0.6 mmol/L.

Experimental Grouping and Treatment

Before modeling, 6 rats of the same age were randomly

selected as control group. After successful modeling, 12 rats were selected and randomly divided into model group and hydrogen treatment group. The optimal therapeutic regimen determined in the preliminary experiment was used for the hydrogen treatment group, i.e. HRS was intraperitoneally injected (5 mg/kg) daily in 7th and 8th weeks after modeling. For control and model groups, an equal volume of normal saline was injected.

Behavioral Test

Thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were detected using the tail-flick test, hot plate test and Von Frey filaments, respectively. 1) TWL was detected before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection. In the hot-water tail-flick test, the tail was immersed in hot water at 52.5±0.5°C, and the duration (s) from tail immersion to tail flick or struggle was recorded for 3 times at an interval of 15 min. The average was taken as the tail-flick latency (TFL). 2) In hot plate test, the heating temperature of YLS-6B hot-plate analgesia meter was set at 52±1°C, and the duration from placement of rats on the hot plate to heating response (paw licking, screaming and jumping) was recorded for 3 times at an interval of 10 min. The average was taken as TWL. 3) MWT was detected before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection. After the rats were placed on a mesh plane for 30 min, MWT (g) of the right hind paw was measured using an electronic Von Frey device 3 times at an interval of 15 min. The average was taken as MWT.

Neurological Function Test

Before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection, the motor nerve conduction velocity (MNCV) of the sciatic nerve was determined. After anesthesia, the rats were fixed in a prone position, and the body temperature was kept at 35-37°C to resist the cooling effect of anesthesia. The sciatic nerve trunk at the ischial tuberosity and hip joint was exposed and separated. The proximal stimulating electrode was placed at the ischiatic notch, the distal stimulating electrode at the medial ankle, the recording electrode at the first femoralis of ipsilateral toes, and the reference electrode between the stimulating electrode and the recording electrode. Upon the stimulation of 3 V single-pulse square wave, the latency of action potential of the proximal and distal sciatic nerves was recorded, and the distance between the two stimulating electrodes and the recording electrode was measured. Finally, MNCV was calculated:
MNCV (m/s) = distance between two stimulating electrodes and recording electrode - action potential latency of two stimulating electrodes.

Sample Collection

After the last behavioral test, the rats were deeply anesthetized with 10% chloral hydrate (300-350 mg/kg), and the thoracic cavity was quickly cut open to expose the heart. The heart was held using a tweezer in the left hand, and the perfusion needle was inserted into the cardiac apex upwards till the ascending aorta. At the same time, a small incision was made using scissors at the right auricle, from which the normal saline was perfused quickly until the outflow liquid was colorless (about 120 mL). In a prone position, the limbs were fixed, the superficial skin was cut open along the femur, and the muscle was bluntly separated to expose the femur. The intermuscular fascia was separated to find the sciatic nerve trunk. The bilateral sciatic nerves were taken, frozen in liquid nitrogen and thawed repeatedly, and its weight was measured. Then the nerves were added with pre-cooled phosphate buffer containing PMSF and protease inhibitor, and homogenized, followed by centrifugation at 12.000 xg and 4°C for 20 min. Finally, the supernatant was stored at -80°C.

Measurement of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) Levels by ELISA

ABC-ELISA was performed. Anti-rat TNF-α (Cat. No. ab208348, Abcam, USA) and IL-6 monoclonal antibodies (Cat. No. ab100772, Abcam, USA) were coated on an ELISA plate, and bound to TNF-α and IL-6 in the standards and samples. Then biotinylated TNF-α and IL-6 antibodies were added, forming an immune complex on the plate. Afterwards, HRP-labeled streptavidin was added to bind biotin, and the substrate solution was used for color development. Finally, sulfuric acid was added to terminate the reaction. The optical density (OD) at 450 nm was measured, which was directly proportional to the concentration of TNF-α or IL-6. The concentrations of inflammatory factors in samples were detected according to the standard curve.

Detection of MCP1 and CCR2 Protein Expressions in the Sciatic Nerve by Western Blotting

Tissue blocks stored in liquid nitrogen were cut into pieces, added with RIPA lysis buffer and PMSF, homogenized on ice and lysed for 30 min, followed by centrifugation at 14.000 rpm and 4°C for 10 min. The supernatant was collected into a centrifuge tube, and the protein concentration was measured using the BCA method. The samples were mixed with loading buffer (1:3), heated at 100°C and cooled to room temperature, followed by centrifugation at 14.000 rpm for 5 min. The supernatant was loaded (10 μL/well), and 5 μL of marker protein was used for labeling. Then the protein sample was subjected to electrophoresis. Subsequently, the product was transferred onto a PVDF membrane which was blocked with skimmed milk on a shaker for 1 h, and incubated with MCP1 (1:500 diluted; Cat. No. ab9858, Abcam, USA), CCR2 (1:500 diluted; Cat. No. ab203128, Abcam, USA) and β-actin (1:500 diluted; Cat. No. ab8226, Abcam, USA) primary antibodies at 4°C

overnight. After the membrane was washed with TBST at room temperature for 5 min (5 times in total), the protein was incubated again with goat anti-rabbit secondary antibody (1:5000) at room temperature on the shaker for 1 h. After the membrane was washed again with TBST for 5 min (5 times in total), ECL solution (solutions A and B mixed at 1:1; Cat. No. ab65623, Abcam, USA) was added (0.125 mL/cm^2) for 2-3 min of reaction, followed by exposure in a darkroom. Finally, OD of protein band was analyzed using Quantity One, and the relative expression level of target protein was expressed as the ratio of its gray value to that of β-actin. The experiments were performed in triplicate independently.

Statistical Analysis

All data were statistically analyzed by SPSS 19.0 software. The quantitative data were represented as mean ± standard deviation. Repeated-measures analysis of variance was performed for intergroup comparisons at different time points. First, the differences between two groups and the time differences of measured values were compared. In the case of intergroup difference, further comparison at each time point was carried out by the independent-sample t test. The SNK-q test was employed to compare the time differences of each group. P<0.05 was considered statistically significant.

Results

Changes of Blood Glucose Level and Body Weight

Compared with the control group, the model group suffered from polyphagia, polydipsia, polyuria, no weight gain and obvious elevation of blood glucose level (P<0.05), gradually became thin and had lusterless hair. The body weight and blood glucose level of diabetic rats were not significantly improved by hydrogen treatment *(Fig. 1)*, indicating that hydrogen cannot inhibit the progression of diabetes by lowering blood sugar.

Behavioral and Neurological Function Test Results

Compared with the baseline values (W0), MNCV, TFL, TWL and MWT of the control group were similar (P>0.05). In the model group, MNCV, TFL, TWL and MWT significantly decreased (P<0.05), and neurological impairment and hyper-algesia occurred from the end of the 2nd week till the 8th week, which were aggravated progressively.

Compared with the model group, the hydrogen treatment group had significantly increased MNCV, TWL and MWT (P<0.05) *(Table 1)*, suggesting that hydrogen effectively elevated MNCV and relieved hyperalgesia in diabetic rats.

TNF-α and IL-6 Levels in Sciatic Nerve

Eight weeks after STZ injection, the levels of TNF-α and IL-6 in the sciatic nerve of the model group were significantly higher than those of the control group (P<0.05), which were significantly lower in the hydrogen treatment group (P<0.05) *(Fig. 2)*. Therefore, hydrogen treatment prevented diabetes-induced elevation of levels of inflammatory factors TNF-α and IL-6 in the sciatic nerve. In other words, inflammatory response was involved in the generation of peripheral neuroinflammatory responses to diabetes, and hydrogen treated neuropathic pain by mitigating inflammation damage.

Effects of Hydrogen on MCP1 and CCR2 Protein Expressions in Sciatic Nerve

Western blotting showed that the protein expressions of MCP1 and CCR2 in the sciatic nerve of the model group were significantly up-regulated compared with those of the control group 8 weeks after STZ injection, suggesting that the MCP1-CCR2 pathway was activated in diabetic

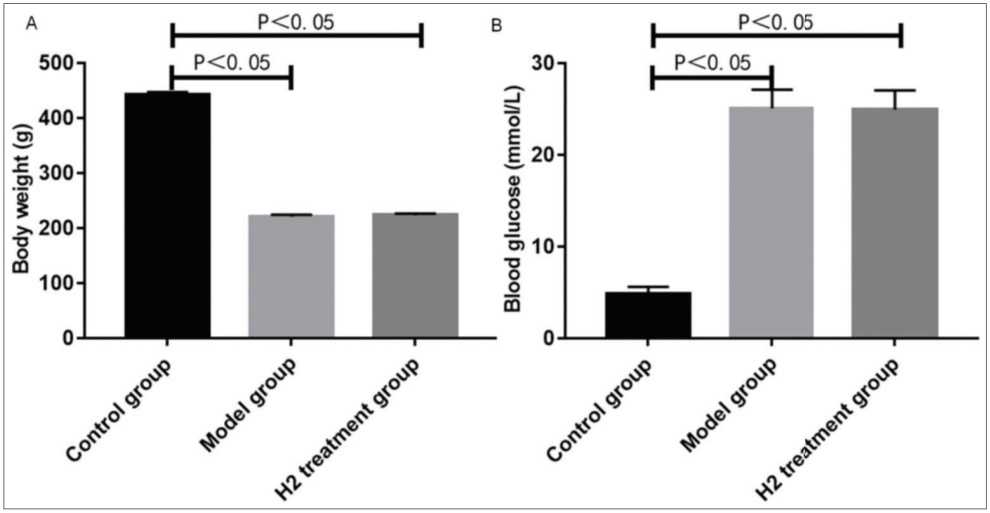

Fig 1. Changes of body weight (A) and blood glucose level (B)

rats. The protein expressions of MCP1 and CCR2 in the hydrogen treatment group were lower than those of the model group *(Fig. 3)*. Based on the results of behavioral experiments, it was found that the activation of the MCP1-CCR2 pathway is consistent with the production of pain sensitivity. Accordingly, hydrogen may alleviate the symptoms of DPN by inhibiting the activation of the MCP1-CCR2 pathway.

Table 1. *Behavioral and neurological function test results*

Time	MNCV (m/s)			TFL (s)			TWL (s)			MWT (g)		
	Control	Model	Hydrogen Treatment	Control	Model	Hydrogen Treatment	Control	Model	Hydrogen Treatment	Control	Model	Hydrogen Treatment
W0	57.4±2.0	56.3±2.9	-	11.7±0.9	11.3±0.9	-	15.8±1.1	15.4±1.0	-	88.0±1.4	88.1±1.5	-
W2	55.2±3.0	38.3±2.0*	-	10.8±0.9	8.5±0.9*	-	15.4±1.0	11.4±0.9*	-	89.0±1.5	68.2±1.5*	-
W4	55.3±2.5	31.7±2.0*	-	11.2±0.9	6.8±0.8*	-	15.3±1.0	8.3±1.0*	-	87.2±1.4	53.2±1.4*	-
W6	56.1±2.4	27.9±2.6*	-	10.5±0.3	5.3±0.7*	-	15.7±1.1	6.8±1.0*	-	87.2±1.9	49.1±1.4*	-
W8	55.8±3.1	29.8±2.3*	48.9±2.3#	11.0±0.8	5.1±0.6*	9.8±0.8#	15.5±1.2	6.2±1.0*	12.7±1.2#	88.2±1.4	49.6±1.37*	7.2±1.5#

** Compared with control group, P<0.05; #compared with model group, P<0.05*

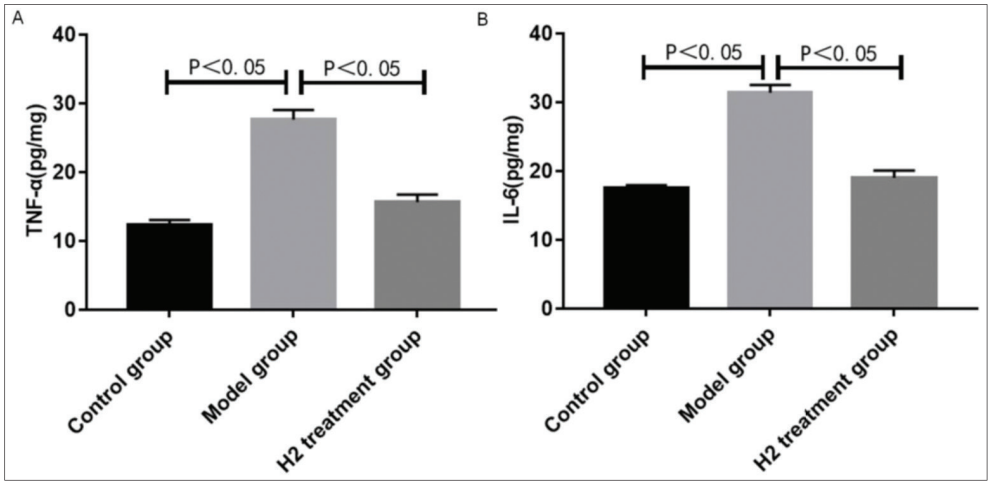

Fig 2. TNF-α (A) and IL-6 (B) levels in sciatic nerve

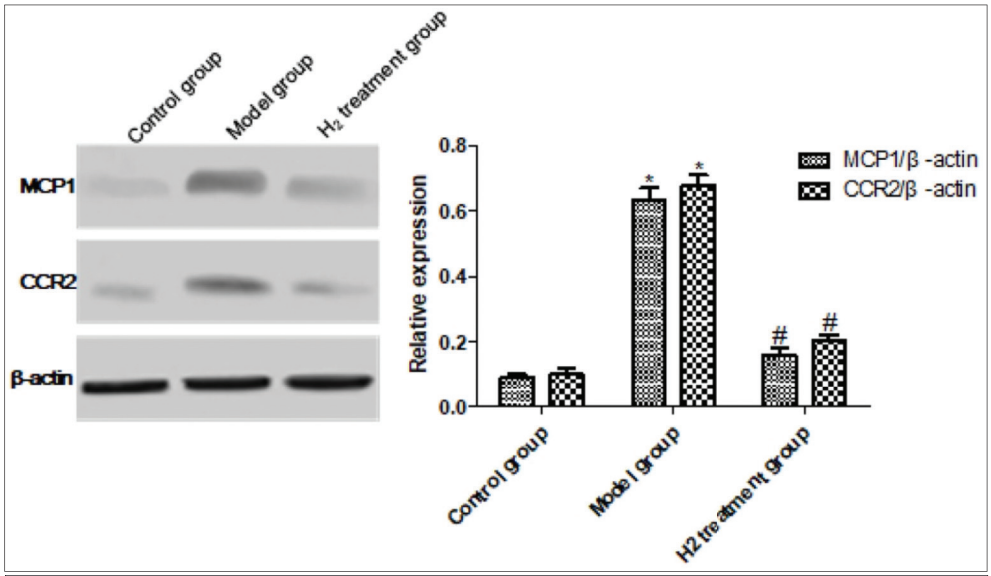

Fig 3. Effects of hydrogen on MCP1 and CCR2 protein expressions in sciatic nerve. * Compared with control group, P<0.05; #compared with model group, P<0.05

Discussion

The incidence of diabetes mellitus, a worldwide epidemic, has increased by 50% in the past decade. The complications of vascular damage caused by chronic hyperglycemia can be divided into two types: microvascular and macrovascular. The microvascular complications include diabetic neuropathy, retinopathy and nephropathy. Diabetic neuropathy is the most common and refractory complication of diabetes, affecting more than half of diabetic patients, of which nearly 15% are at risk of lower limb amputation, or even threatening lives. The early manifestations of pain symptoms are spontaneous pain, hyperalgesia and paralgesia. In the later stages, the symptoms are manifested as hypoalgesia decrease, which seriously affects the quality of life of patients. Due to the complex pathological mechanism of DPN, there is still no effective treatment. STZ is a toxin that specifically destroys islet cells. A single injection can lead to sudden hyperglycemia. The model of diabetes induced by STZ is the most classic model for DPN research. STZ models usually show the changes of pain behavior, nerve function and nerve morphology that are basically consistent with those of diabetic patients. Early neurological symptoms are usually characterized by time-dependent aggravation of hyperalgesia, paralgesia, and decreased nerve conduction velocity, while late neurologic symptoms are manifested as hypoalgesia, neuro-degeneration, demyelination and loss of epidermal nerve fibers. Obvious hyperalgesia and paralgesia usually occur two weeks after STZ injection, and can continue to aggravate to the eighth week. After eight weeks, the symptoms such as hypoalgesia appear, which may be related to the irreversible degeneration of nerve fibers. Nerve function indicators include motor nerve conduction velocity and sensory nerve conduction velocity, which are currently considered as the gold standard for evaluating the degree of nerve damage. Their decline is the manifestation of nerve hypoxia and ischemia, which is more sensitive and objective than the change of pain threshold in the study of nerve injury [8,9]. In this study, a model was established with reference to literature methods, and the results were consistent with the above theory. Compared with the control group, the rats appeared to have diabetic symptoms such as polyphagia, polydipsia and polyuria 48 h after STZ injection, and their blood glucose increased to above 16.67 mmol/L. The results of the behavioral and neurological experiments showed that the mechanical pain threshold and thermal pain threshold decreased significantly, and the sciatic nerve conduction velocity reduced significantly 14 days after STZ injection in a time-dependent manner until the eighth week after injection, indicating that DPN was successfully induced and continued to develop.

The long course of diabetes and poor glycemic control are the main risk factors for neuropathy. Due to the lack of understanding of its pathogenesis, there is still no effective treatment except for the control of blood glucose and pain treatment. Some studies have found that even the strict control of blood glucose cannot prevent the development of DPN, indicating that hyperglycemia is only a pathogenic inducer, and DPN can also play a damaging role through a variety of mechanisms at the downstream [10,11]. In addition, because the development of microvascular complications is a process from reversibility to irreversibility, it is of great significance to effectively control and reverse the disease by finding the early markers of its onset or pathogenic causes and dealing with them.

Neuropathic pain refers to the type of pain in which a nociceptive response still exists after the noxious stimulus to nerves and surrounding tissues is removed. It is mainly manifested in overreaction to harmful stimulus or abnormal response to mild stimulus. Postherpetic neuralgia trigeminal neuralgia and DPN are the three most common types in clinical practice. Neuropathic pain is still regarded as one of the most difficult types of chronic pain to deal with, which seriously affects the quality of life of patients and brings a huge burden to society. A variety of neural injury models have confirmed that the production of pain symptoms is closely related to the inflammatory response. It has been found in clinical studies that the levels of inflammatory factors in the blood of patients with type 1 or type 2 diabetes were higher than those of healthy people, and this increase often indicated the occurrence and progression of neuropathy [12]. Increased levels of inflammatory factors also play an important role in the production and maintenance of DPN. Drug and gene intervention studies have also proven that the use of infliximab or knockout of TNF-α gene can improve pain symptoms and neurological function in diabetic mice [13]. Etanercept, a selective inhibitor of TNF-α, is a kind of human recombinant soluble TNF-α receptor. 2 weeks of etanercept treatment for diabetic rats modeled for 6 weeks can significantly improve thermal hyperalgesia and motor nerve conduction velocity, which indicate that the inflammatory response may be involved in the occurrence of DPN. In this study, TNF-α and IL-6 levels in the sciatic nerve of rats in the DPN group were both higher than those in healthy rats, which also verified this hypothesis. Molecular hydrogen has been successfully used in a variety of acute oxidative stress environments due to its effective antioxidant and free radical scavenging effects. Its main molecular targets are not yet clear. At present, the most important mechanism is the selective scavenging of free radicals and peroxynitrite. Moreover, it also has the role of regulating gene expression [14]. Hydrogen inhalation can significantly reduce the levels of serum and tissue oxidation products, improve the activity of

antioxidant enzymes, increase the survival rate of mice with moderate or severe sepsis, reduce multiple organ damage caused by sepsis, and effectively prevent the occurrence of multiple organ failure [15,16]. Meanwhile, no obvious side effects were found by detecting various physiological indices of animals in the process of hydrogen treatment. In addition to direct anti-oxidative stress and scavenging free radicals, the main mechanism of its action can also play a further therapeutic role by down-regulating the expression of inflammatory protein and up-regulating the expression of antioxidant protein *in vivo*. Herein, HRS inhibited the aggravating symptoms and functional impairment of neuropathic hyperalgesia in diabetic rats, and the effect was correlated with the decrease of proinflammatory cytokine levels. Therefore, we postulated that molecular hydrogen may play a protective role in the chronic oxidative stress environment of the nervous system and the inflammatory damage caused by it, so as to treat DPN.

Hyperglycemia causes damage through a variety of mechanisms, involving multiple damages to the metabolic environment, nerves and blood vessels: it can lead to polyol bypass activation, excessive production of terminal glycosylation products, excessive activation of the MCP1-CCR2 pathway, inflammatory response, and mitochondrial damage. These mechanisms, through their own effects or mutual effects, can eventually lead to the excessive production of ROS and the breakdown of the body's redox balance, resulting in oxidative stress. Thus, oxidative stress is the common result of these pathways and plays an important role in the formation of DPN. Since neuropathic pain in diabetic rats is closely related to neuroinflammatory response, and the MCP1-CCR2 pathway is an important regulator of DPN inflammatory response [17], this study aimed to assess the effect of HRS on the MCP1-CCR2 pathway. Six weeks after STZ injection, the levels of MCP1 and CCR2 in the sciatic nerve of the DPN group were significantly down-regulated by continuous injection of HRS for 14 days, the content of proinflammatory cytokines regulated by STZ was also decreased, the thermal and mechanical pain thresholds were significantly increased, and the nerve conduction velocity was improved.

In summary, hydrogen may reduce the inflammatory response of peripheral nerves in diabetic rats by inhibiting the activation of the MCP1-CCR2 signaling pathway, thereby improving neuralgia. Due to the complexity of the pathogenesis of DPN and the limited therapeutic strength for one mechanism alone, it is necessary to find a comprehensive treatment that can inhibit the etiology, control the inflammation and strengthen the self-defense mechanism so as to prevent and recover the nerve damage and resolve pain.

Availability of Data and Materials

All data and materials are available from the corresponding authors upon reasonable request.

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Competing Interests

There is no conflict of interest.

Authors' Contributions

PW, LY and QL designed this study and prepared this manuscript; HW, YL and WC performed this study and analyzed experimental data. All authors read and approved the final version of the manuscript.

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Research Article

Effects of Supplementation with Rumen-Protected Methionine on Milk Performance, Plasma Biochemical Indices and Amino Acid Concentration in Dairy Goats Subject to Heat Stress

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Abstract: This study examined the effects of four levels of rumen-protected methionine (RPM) on the milk performance of dairy goats subjected to severe heat stress (temperature humidity index >90). Seventy-five Guanzhong dairy goats (52.6±4.9 kg) with the same farrowing period and lactation day (120±10 d) were randomly divided into five groups of 15 head and fed a 13.55% crude protein diet supplemented with 0, 1.5, 3, 4.5 or 6 g/d RPM in the concentrate. The trial included nine days adaptation time and 30 days of sampling and analysis. The addition of RPM did not change feed intake but 1.5 g/d RPM significantly increased milk protein content, the ratio of milk to feed, and the economic returns. No significant changes in milk fat or urea-nitrogen concentrations or somatic cell count were observed. Plasma urea-nitrogen was significantly lowered in the 1.5 g/d RPM group. RPM supplementation increased plasma methionine concentration and total amino acid concentration in a dose-dependent manner. The highest dose of RPM (6 g/d) enhanced plasma immunoglobulin G concentration. It is demonstrated that supplementation with an appropriate dose of RPM to dairy goats fed a relatively low protein diet and subject to heat stress can increase milk protein production and improve economic returns.

Keywords: *Rumen protected methionine, Milk composition, Plasma urea-nitrogen, Free amino acids, Immunoglobulin G, Dairy goats*

Isı Stresine Maruz Kalmış Süt Keçilerinde Rumen Korumalı Metiyonin İlavesinin Süt Performansı, Plazma Biyokimyasal İndeksleri ve Amino Asit Konsantrasyonu Üzerine Etkileri

Öz: Bu çalışmada, şiddetli ısı stresine (sıcaklık-nem indeksi >90) maruz kalan süt keçilerinde rumen korumalı metiyoninin (RPM) dört farklı konsantrasyonunun süt performansı üzerine etkisi incelendi. Aynı yavrulama zamanı ve laktasyon dönemine (120±10 gün) denk gelen 75 Guanzhong süt keçisi (52.6±4.9 kg), 15 hayvandan oluşan beş gruba ayrıldı ve her bir grup 0, 1.5, 3, 4.5 ve 6 g/gün konsantreli RPM içeren %13.55 ham protein diyeti ile beslendi. Deneme, dokuz günlük adaptasyon süresi ve 30 günlük örneklem ve analiz süresini içeriyordu. RPM ilavesi yem tüketimini değiştirmez iken, 1.5 g/gün konsantreli RPM ilavesi süt protein içeriğini, sütün besleme oranını ve ekonomik kazancı önemli ölçüde artırdı. Süt yağı ve üre-azot konsantrasyonlarında ve somatik hücre sayısında önemli bir değişiklik gözlenmedi. 1.5 g/gün konsantreli RPM uygulanan grupta plazma üre-azot seviyesi önemli ölçüde azaldı. RPM ilavesi, doza bağımlı bir şekilde plazma metiyonin ve toplam amino asit konsantrasyonunu arttırdı. RPM'nin en yüksek dozu (6 g/gün), plazma immünoglobulin G konsantrasyonunu artırdı. Nispeten düşük protein diyeti ile beslenen ve ısı stresine maruz kalan süt keçilerine uygun dozlu RPM ilavesinin, süt protein üretimini artırabileceği ve ekonomik kazancı iyileştirebileceği gösterilmiştir.

Anahtar sözcükler: *Rumen koruyucu metiyonin, Süt bileşimi, Plazma üre-azot, Serbest amino asitler, İmmünoglobulin G, Süt keçisi*

Introduction

Low protein utilization and lack of protein-rich feed make ruminant production less economical. It is necessary to improve the utilization of protein by ruminants [1] the limiting of supplemental amino acids being an important approach [2]. Heat stress is a series of non-specific physiological reactions in animals subject to high

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temperature and high humidity environments, and is an important factor hindering the development of the dairy goat industry due to its adverse effects on milk production and composition [3-5]. Studies have shown that when animals are subjected to heat stress their feed intake, reproductive performance, and productivity are affected, and, in severe cases, death can result [6]. Heat stress affects immune function at the interface of the endocrine and immune systems. Lactating ewes under heat stress have shown elevated mastitis pathogen loads [7]. Thus, the alleviation of heat stress and improvement of ruminant performance must be urgently addressed.

Methionine (Met) is considered to be the primary limiting amino acid in milk production in dairy ruminants [8,9]. It is an effective regulator of protein synthesis [10,11] and involved in transsulfuration, methylation reactions and polyamine synthesis [11]. Met has a significant impact on oxidative stress status because it is essential for the synthesis of glutathione, one of the most abundant antioxidants produced in the liver [12]. So, the essential amino acid methionine is one of the antioxidant nutrients that act to mitigate the deleterious effects of ROS to cells. Thus, in addition to involvement in glutathione synthesis, Met supplementation has a direct protective effect against oxidative stress in animals under heat stress. Its functions include the maintenance of animal growth, the development of physiological activities, detoxification and anti-mould activities, and myocardial protection. Insufficient Met can cause animal weight loss, stunted growth, liver and kidney dysfunctions, and poor milk quality [13].

Dietary Met can be rapidly degraded by microorganisms in the rumen, while Met in the microbial proteins that enter the small intestine of the host is usually insufficient for high milk production [14]. Previous studies have shown that supplementing the feed of lactating ruminants with rumen-protected methionine (RPM) consistently increased milk protein concentration and milk protein yield [15-18]. Supplementation of dairy cow diets with RPM can improve the utilization efficiency of feed protein, fulfil protein requirements for lactation and reduce the influence of heat stress on milk yield. Zhao et al.[19] compared a low-protein diet (12% CP) supplemented with RPM to a high-protein diet (16% CP). The supplemented low-protein diet increased both milk protein yield and urea-nitrogen content and tended to increase milk yield. Lee et al.[20] reduced the dietary protein of dairy cows from 15.7% to 13.5% and found a significant reduction in milk yield that could compensated by supplementation with RPM. Many reports have revealed that supplementation with RPM increased milk production and immunity of lactating cows [21,22], but there have been few studies on RPM supplementation of dairy goats.

The main hypothesis of this study is that adding RPM to a low-protein diet increases plasma Met concentrations, promotes lactation, improves the utilization of dietary protein by dairy goats, and alleviates the effects of heat stress. The National Research Council (NRC) [23] state that it is common to supplement dairy goats with Met, but the optimal dose of RPM is unknown. In this study various doses of RPM were given to dairy goats and the effects on milk quantity and quality and blood indices were assessed, in order to define the appropriate RPM dose for dairy goats subject to heat stress.

Material and Methods

Source of RPM

RPM containing 57% 2-hydroxy-4-(methylthio)isopropyl butyrate was purchased from Adisseo Life Science Products Co., Ltd. (Shanghai, China).

Animals and Experimental Design

The trial was conducted at Taiping Farm (Shaoyang, Hunan, China; latitude 27°14'N, longitude 111°27'E). Seventy-five Guanzhong dairy goats, a local Chinese breed, with similar live weights (52.6±4.9 kg) and at similar lactation ages (120±10 d) were selected and allocated to five group consisting of 15 head per group. All animals were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the College of Animal Science and Technology, Hunan Agricultural University (CASTHAU-02-2019-10). The groups were randomly assigned to five treatments involving supplementation of the basal diet with RPM at doses of 0 (control group), 1.5, 3.0, 4.5, and 6.0 g/d head. The trial lasted for 39 days, consisting of nine days for acclimation and 30 days (the experimental period) for sampling and analyses.

Diet and Animal Management

The diet referenced NRC nutrient requirements for dairy goats [23] and was prepared as a total mixed ration. Dietary ingredients and nutritional parameters are listed in *Table 1*. The average CP content of the basal diet in this study (13.55%) was lower than the CP content recommended by the NRC [23]. Diet with low protein level (13.55%) was fed to groups in the current study. Goats were kept in individual pens and fed twice daily at 08:00 and 18:00. Fresh drinking water was available all times.

Measurement of Temperature-Humidity Index (THI)

Wet-bulb and dry-bulb thermometers were hung in the barn 1.5 m above the ground. Temperature and humidity were recorded every day at 08:00, 12:00, and 17:00. The formula THI = 0.72 x (Td + Tw) + 40.6 was applied, where Td represents dry-bulb temperature and TW represents wet-bulb temperature [24]. The average of the two thermometer readings was recorded. As shown in

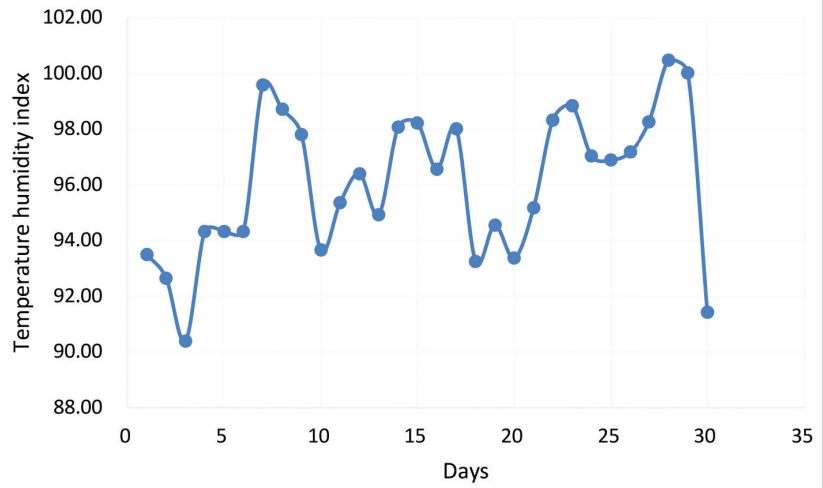

Fig 1. The temperature-humidity index figure during the experimental period

Table 1. *Composition and nutritional parameters of the basal diet (dry matter basis)*

Ingredients	%	Nutritional Parameters	%
Alfalfa hay	24.00	DM	92.94
Oat hay	24.00	CP	13.55
Alfalfa pellets	12.00	NDF	52.75
Corn	27.72	ADF	21.96
Wheat middling	2.00	Ca	0.39
Soybean meal	9.30	P	0.26
$Ca(HCO_3)_2$	0.60	Met	0.24
NaCl	0.30		
$CaCO_3$	0.03		
Premix[1]	0.05		
Total	100.00		

[1] Premix provides (Purina commercial feed, per kg feed, dry matter basis): Vit. A- 15.544 IU, Vit. D- 23.220 IU, Vit. E- 297 IU, Fe- 30 mg, Cu- 25 mg, Zn- 60 mg, Mn- 75 mg, I- 0.15 mg, Se- 0.05 mg, Co- 0.15 mg
DM: *dry matter;* ***CP:*** *crude protein;* ***NDF:*** *neutral detergent fiber;* ***ADF:*** *acid detergent fiber;* ***Ca:*** *calcium;* ***P:*** *phosphorus;* ***Met:*** *methionine*

Fig. 1, the THI during the experimental period was >90, indicating the animals were subject to severe heat stress [25].

Sampling and Analyses

The quantities of feed offered and refused were recorded daily during the experimental period to calculate the average daily feed intake. Samples of the ration and any feed refused were taken daily and stored at -20°C. At the end of the experimental period, feed samples were pooled, subsampled, and dried at 65°C for 48 h. Dried samples were ground through a 40-mesh sieve and their chemical composition determined using methods described by the Association of Official Analytical Chemists [26].

Goats were milked twice per day during the experimental period using a parallel milking machine (DeLaval Co., Ltd., Sweden) and milk yield was recorded. Milk samples (200 mL stored at -20°C) were taken on the last day from nine randomly selected goats in each group. These were aliquoted into 4 x 50 mL centrifuge tubes for milk composition determination. There were three replicates per sample. Concentrations of fat, protein, and non-fat milk solids were determined using a MILKANA Milk Analyzer (Beijing Harold Technology Co., Ltd., China). Somatic cell count (SCC) was determined using a Lilavar SCC analyzer (Nova Zagora, Bulgaria). Urea-nitrogen was determined with an enzymatic (urease) method using a commercial kit (Nanjing Jiancheng, China) following the manufacturer's protocol.

Feed costs, gross income from milk, and economic return from the milk (i.e., ratio of milk income to the feed cost) were calculated as follows:

Feed costs (¥/d) = Feed intake (kg/d) x Feed price (¥1.68/kg) + RPM cost (¥75/kg)

Milk income (¥/d) = Milk yield (kg/d) x Milk price (¥7.50/kg)

Gross milk income (¥/d) = Milk income - Feed cost

where ¥ is Renminbi (RMB). The ratio of average milk yield (kg/d) to average feed intake (kg/d) was also calculated.

On day 30 of the experimental period, nine goats from each group were randomly selected for blood sampling before morning feeding. A total of 15 mL of blood was drawn from the jugular vein and plasma was obtained by centrifugation at 1.600 x g for 20 min and stored at -20°C. Plasma concentrations of urea-nitrogen, glucose, triglycerides, total cholesterol, and immunoglobulin G were determined using commercial kits (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) following the manufacturer's procedures. Free amino acid concentrations were determined by a high-performance liquid chromatography (LC-10ADvp equipped with Fr-10ADvp; Shimadzu, Japan) according to the method of Hughes et al.[27].

Statistical Analysis

Differences between the five trial groups were analyzed by one-way ANOVA using SAS 9.4 software [28]. Since four doses of RPM were offered, the linear, quadratic and cubic trends were tested using CONTRAST statements with orthogonal polynomial coefficients estimated by PROC IML. Data are presented as least square means and standard error of the means. Differences between means with P≤ 0.05 were considered statistically significant.

Results

Effects of RPM on Milk Performance and Economic Return

As shown in *Table 2,* there were no significant differences in dry matter intake or milk yield between the groups (P>0.05). However, milk yield showed a significant quadratic increase (P<0.05) in the treatment groups compared with the control group. The milk to feed ratio was higher in the 1.5, 3, and 4.5 g/d RPM groups than in the control and 6 g/d RPM groups (P<0.05) while quadratic and cubic effects were evident (P<0.05).

Protein and non-fat solids concentrations were highest in the 6 g/d RPM group, followed by the 1.5 g/d RPM group. Both concentrations were significantly higher in these groups than in the other three groups (P<0.05), and exhibited significant cubic responses (P<0.05). No significant differences between groups were found for milk fat and urea-nitrogen concentrations and SCC count (P>0.05), but milk fat in RPM-supplemented groups was higher than in the control group.

There were no significant differences in milk income among groups (P>0.05). However, milk income showed a significant quadratic trend (P<0.05), being higher in the treatment groups than the control group. Gross profit showed linear, quadratic and cubic relationships with RPM dose and was higher in the 1.5 and 3 g/d RPM groups than in the other groups (P<0.05). Feed cost increased with the quantity of RPM supplement and displayed linear, quadratic, and cubic relationships (P<0.05).

Effects of RPM on Plasma Biochemical Parameters

Plasma urea-nitrogen concentration was lower in the treatment groups than the control group, with the 1.5 g/d group being significantly lower than other treatment groups (P<0.05). There were no significant differences between the groups in plasma concentrations of glucose, non-esterified fatty acids, total cholesterol, and total triglycerides (P<0.05; *Table 3),* but total cholesterol and total glycerides were lower in the treatment groups than in the control group. Immunoglobulin G was highest in the 6 g/d RPM group (P<0.05), and showed linear, quadratic, and cubic relationships (P<0.05).

Effects of RPM on Plasma Free Amino Acid Concentration

Table 4 shows plasma concentrations of essential and non-essential amino acids. Supplementation with RPM increased Met concentration in a dose-dependent manner, the increments appearing between the 0 to 4.5 g/d RPM groups but being much greater in the 6 g/d group.

Generally, RPM supplementation increased the total essential amino acid (TEAA) concentration in plasma, but this effect varied between RPM doses. The greatest

Table 2. *Effects of supplementation with rumen-protected methionine on milk production and economic returns in dairy goats*

Item	Rumen-Protected Methionine Supplement (g/d)					SEM	P-value		
	0	1.5	3	4.5	6		Linear	Quadratic	Cubic
DMI, kg/d	2.65	2.64	2.70	2.63	2.67	0.021	0.607	0.774	0.791
Milk yield, kg/d	0.79	0.85	0.87	0.84	0.80	0.030	0.835	0.048	0.107
Milk-feed ratio	0.30^{a}	0.32^{b}	0.32^{b}	0.32^{b}	0.30^{a}	0.003	0.666	<0.001	<0.001
Milk Composition									
Fat, %	3.11	3.26	3.43	3.70	3.27	0.184	0.230	0.159	0.173
Protein, %	3.05^{a}	3.20^{b}	3.06^{a}	3.07^{a}	3.21^{b}	0.048	0.252	0.409	0.032
SNF,%	8.07^{a}	8.46^{b}	8.09^{a}	8.10^{a}	8.51^{b}	0.134	0.293	0.445	0.036
MUN, mmol/L	3.21	3.34	3.55	3.86	3.28	0.184	0.400	0.220	0.184
SCC, ×10^{4}/mL	23.84	24.41	24.25	39.48	29.67	0.353	0.247	0.514	0.504
Economic Returns									
Milk income, ¥/head·d	5.55	5.93	6.07	5.86	5.58	0.212	0.865	0.048	0.107
Feed cost, ¥/head·d	4.44^{a}	4.56^{b}	4.76^{c}	4.76^{c}	4.94^{d}	0.036	<0.001	<0.001	<0.001
Gross profit, ¥/head·d	1.11^{b}	1.37^{c}	1.31^{c}	1.10^{b}	0.64^{a}	0.035	<0.001	<0.001	<0.001

DMI: *dry matter intake;* ***Milk income:*** *milk yield x milk price;* ***MUN:*** *milk urea-nitrogen;* ***SCC:*** *somatic cell count;* ***SNF:*** *non-fat milk solids;* ***¥:*** *Chinese Renminbi; Mean values within a row with different superscript letters differ significantly (P<0.05)*

Table 3. Effects of supplementation with rumen-protected methionine on plasma biochemical indices in dairy goats

Item	Rumen-Protected Methionine Supplement (g/d)					SEM	P-value		
	0	1.5	3	4.5	6		Linear	Quadratic	Cubic
PUN, mmol/L	6.31[b]	5.08[a]	6.09[b]	5.91[b]	6.03[b]	0.264	0.861	0.647	0.423
GLU, mmol/L	1.83	1.60	1.67	1.64	1.62	0.141	0.448	0.667	0.774
IgG, mg/mL	9.42[a]	9.24[a]	9.31[a]	9.26[a]	10.69[b]	0.243	0.048	0.014	0.023
NEFA, mmol/L	0.25	0.22	0.27	0.25	0.23	0.021	0.929	0.781	0.586
TC, mmol/L	2.99	2.57	2.76	2.58	2.46	0.145	0.068	0.187	0.234
TG, mmol/L	0.30	0.22	0.29	0.24	0.28	0.023	0.997	0.384	0.486

PUN: *plasma urea-nitrogen;* ***GLU:*** *glucose;* ***IgG:*** *immunoglobulin G;* ***NEFA:*** *non-esterified fatty acids;* ***TC:*** *total cholesterol;* ***TG:*** *triglycerides; Mean values within a row with different superscript letters differ significantly (P<0.05)*

Table 4. Effects of supplementation with rumen-protected methionine on plasma free amino acid concentrations (mg/100 mL) in dairy goats

Item	Rumen-Protected Methionine Supplement (g/d)					SEM	P-value		
	0	1.5	3	4.5	6		Linear	Quadratic	Cubic
EAA									
Met	0.37[a]	0.51[b]	0.67[c]	0.84[d]	1.40[e]	0.050	<0.001	<0.001	<0.001
Lys	1.72[a]	2.12[b]	2.54[c]	2.12[b]	2.12[b]	0.120	0.009	0.006	0.012
Leu	1.98[a]	1.85[a]	2.29[a]	2.16[a]	3.67[b]	0.150	<0.001	<0.001	<0.001
Ile	1.78[a]	1.35[a]	2.65[b]	2.69[b]	4.84[c]	0.175	<0.001	<0.001	<0.001
His	0.70[a]	0.83[ab]	1.37[c]	0.96[b]	1.45[c]	0.079	<0.001	<0.001	<0.001
Arg	2.72	3.00	2.94	2.52	2.76	0.153	0.560	0.633	0.256
Phe	1.30[a]	1.62[a]	2.25[b]	2.28[b]	2.46[b]	0.110	<0.001	<0.001	<0.001
Thr	5.68[a]	7.38[b]	9.25[d]	6.88[b]	8.27[c]	0.248	0.004	0.001	<0.001
Val	3.62[a]	4.22[a]	5.46[b]	5.66[b]	8.38[c]	0.220	<0.001	<0.001	<0.001
BCAA	7.58[b]	7.11[a]	10.58[c]	10.29[c]	17.90[d]	0.110	<0.001	<0.001	<0.001
TEAA	20.04[a]	22.20[b]	30.91[d]	25.06[c]	37.28[e]	0.324	<0.001	<0.001	<0.001
NEAA									
Ala	2.87[a]	3.27[a]	5.88[b]	6.19[b]	10.92[c]	0.215	<0.001	<0.001	<0.001
Asp	0.15	0.12	0.14	0.14	0.15	0.015	0.601	0.592	0.689
Glu	2.60[a]	2.44[a]	3.45[b]	2.82[a]	3.88[b]	0.162	<0.001	0.001	0.003
Ser	1.00[a]	1.11[a]	1.83[b]	1.71[b]	2.73[c]	0.104	<0.001	<0.001	<0.001
Gly	0.67[a]	1.83[b]	2.39[c]	2.06[b]	2.45[c]	0.106	<0.001	<0.001	<0.001
Tyr	0.79[a]	1.44[b]	2.19[d]	1.79[c]	2.12[d]	0.105	<0.001	<0.001	<0.001
NEAA	8.07[a]	10.20[b]	16.07[c]	16.22[c]	24.70[d]	0.230	<0.001	<0.001	<0.001
TAA	28.11[a]	32.29[a]	46.99[c]	38.27[b]	59.60[d]	1.905	<0.001	<0.001	<0.001

Ala: *alanine;* ***Arg:*** *arginine;* ***Asp:*** *aspartic acid;* ***BCAA:*** *branched chain amino acids;* ***Glu:*** *glutamate;* ***Gly:*** *glycine;* ***His:*** *histidine;* ***Ile:*** *isoleucine;* ***Leu:*** *leucine;* ***Lys:*** *lysine;* ***Met:*** *methionine;* ***NEAA:*** *non-essential amino acids;* ***Phe:*** *phenylalanine;* ***Ser:*** *serine;* ***TAA:*** *total amino acids;* ***TEAA:*** *total essential amino acids;* ***Thr:*** *threonine;* ***Tyr:*** *tyrosine;* ***Val:*** *valine; Mean values within a row with different superscript letters differ significantly (P<0.05).*

TEAA increment was with the 6 g/d RPM group, followed by the 3 g/d group, then the 4.5 and 1.5 g/d groups *(Table 4)*. Branched-chain amino acids (BCAA) were lowered by supplementation with 1.5 g/d RPM but were significantly increased in the other treatment groups. The effects of RPM supplementation on individual EAAs varied, depending on RPM dose. In general, RPM supplementation increased plasma concentrations of Lys, His, Phe, and Thr, but had no significant influence on Arg concentration.

Supplementation with RPM had no significant influence on Asp concentration in plasma (P>0.05) but increased the concentration of the other non-EAAs (Ala, Glu, Ser, Gly, and Tyr). The total concentration of non-EAAs increased with RPM dose *(Table 4)*.

Total amino acid concentration increased linearly with RPM dose, except for the 4.5 g/d RPM group which was lower than the 3 and 6 g/d groups. In addition, linear, quadratic, and cubic relationships were evident (P<0.05) with Met, Lys, Ile, His, Phe, Thr, Val, BCAA, EAA, Ala, Gly, Ser, Gly, and Tyr, and non-EAA.

Discussion

Goats living under conditions of heat stress with 13.55% crude protein in their diets (i.e., a low-protein diet) produced 0.8-0.9 kg of milk per day. Adding 1.5 or 6 g/d RPM to the diet increased the protein and non-fat solid contents of the milk and improved economic returns. Milk yield showed a significant quadratic increase, being higher in the treatment groups than the control group. Milk fat concentration tended to increase in response to RPM dosage (1.5 to 4.5 g/d) but did not reach statistical significance. The present findings are supported by previous reports. Mateus et al.[29] showed that cows given RPM had increased milk protein (3.07 vs. 2.95%), yield (1.48 vs. 1.43 kg/d), and fat (3.87 vs. 3.77%), although milk fat yield did not change. Similar increases in protein yield and concentration following RPM supplementation have been reported in dairy goats. For example, a 2.5 g/d RPM supplement given to Shami goats from the final 60 days of pregnancy through the first 60 days of lactation was reported to increase milk yield from 1.18 kg/d (control group) to 1.36 kg/d, and milk protein concentration from 3.55% to 3.85%. The Shami goats weighed 47-75 kg and were fed a basal diet containing 17.5% crude protein [30]. In Zaraibe goats (bodyweight ~35 kg) fed a basal diet containing about 14% crude protein, supplementation with 2 g/d RPM increased milk yield from 1.73 kg/d (control group) to 2.07 kg/d. Milk protein increased from 8.67% to 9.24% (RPM group), respectively, SNF also increased correspondingly [31]. Increments in milk yield and protein concentration were also observed in Saanen goats fed a basal diet containing 14.4% crude protein and supplemented with 2.5 g/d RPM [32], and in Alpine dairy goats supplemented with 5 g/d RPM [33]. These effects vary depending on lactation stage, milk yield and dietary crude protein content [13]. Heat stress adversely affects milk production and composition in dairy animals. According to Bouraoui et al.[34], daily THI negatively correlated to milk yield, and lower milk fat and milk protein were observed in the summer season. Hamzaoui et al.[35] also reported milk with lower protein (6-13%) content in heat stressed goats. This demonstrates that heat stress affects the health of dairy animals, impacting their normal physiology and metabolism. The results of this study show that RPM supplementation tends to improve milk yield in dairy goats, possibly because it increases Met supply in the small intestine, and, subsequently, the mammary gland, providing sufficient precursors for protein synthesis in the gland. Thus, RPM supplementation effectively promotes the metabolic balance of amino acids in ruminants, improves the utilization rate of amino acids, and reduces the adverse effects of heat stress, providing adequate energy for milk production and milk protein synthesis, and promoting performance. In summary, supplementation of low-protein diets with RPM alleviates the negative effects of heat stress. Higher doses of RPM yield no further benefits.

The high observed milk to feed intake ratio and the static feed intake in the 1.5 g/d RPM group suggests that the increases in milk protein and non-fat solids resulted from the increased utilization of nutrients, particularly protein. This study did not measure the digestion and metabolism of N in the goats. However, RPM supplementation may increase protein digestibility in some cases, for example in Zaraibe goats [31]. Muramatsu et al.[36] showed that giving RPM to Japanese Saanen goats substantially increased whole-body protein synthesis while reducing urinary N excretion; so N balance and utilization efficiency were markedly increased at a constant N intake [37]. Improved N utilization efficiency in response to RPM supplementation is supported by the reduced plasma urea-nitrogen concentration observed in the current study, plasma urea-nitrogen being a good indicator of protein status [38].

RPM did not alter the plasma concentrations of glucose, non-esterified fatty acids, total cholesterol, or total glycerides, but total cholesterol and total glycerides in the treatment groups were lower than those in the control group. Total cholesterol content reflects the lipid metabolism status of the body. Total glycerides is a product of fat metabolism and reflects the digestion and absorption status of fat-the higher the content, the lower the utilization rate of body fat [39]. This also corresponded with the milk fat of the treatment groups being higher than the control group. The only substantial change was plasma IgG concentration, which was significantly elevated in the 6 g/d RPM group. The immune system is the major body defense systems to protect and cope against environmental stressors [40]. Met participates in the synthesis of immune system molecules (cytokines, antibodies, complement, etc.) [41] and influences the levels of immunoglobulins in the body. In order to enhance the humoral immune response, Met is present at much higher concentrations than required for production and health needs [42]. The health implications for dairy goats of having such high IgG levels in plasma are not clear and the molecular mechanism by which high levels of Met boost IgG concentrations warrants further research.

Plasma concentrations of free amino acids reflect the balance between amino acids entering the metabolic pool from both intestinal absorption and metabolic protein breakdown, and those leaving the pool as a result of protein synthesis and irreversible oxidation [43]. Based only on changes in plasma concentration, it is not possible to judge how this two-way flux of amino acids contributes to shifts in concentration. It is notable that plasma Met concentration increased in response to RPM dose, suggesting that the amount of Met absorbed from the intestine increased as a result of the supplementation, improving lactation

performance and levels of immunity. Dose-responsive changes in plasma Met concentration have been observed in other studies. For example, plasma free Met and the ratio of Met to total essential amino acids showed significant linear responses to RPM supplementation of Merion sheep (1, 2, 3, 5, and 8 g/d) [44,45] and dairy cows [46]. Literature on changes in plasma Met concentrations in dairy goats supplemented with RPM is scarce, so definitive conclusions cannot be drawn. Increases in total EAA and non-EAA plasma concentrations were observed in this study after RPM supplementation. Changes in total plasma amino acids after RPM supplementation of dairy goats and cows vary. No changes [45,46], decreases [47], and increases [48,49] have all been reported. Amino acids not only serve as direct precursors for protein synthesis, but also act as regulators of the rate of protein synthesis. When activated by Met, mammalian target of rapamycin may increase the initiation rate of protein synthesis [50], which might explain the increased milk protein production of RPM-supplemented dairy goats.

The experimental design of the current study did not allow the source of the increase in total plasma amino acids to be defined; it could be attributed to either increased dietary protein digestion or reduced cellular oxidation of amino acids. Further studies are needed to explore the mechanisms of Met-associated metabolic processes.

In conclusion, supplementing the diet of dairy goats with an appropriate dose of RPM (1.5 g/d) while being fed a relatively low protein diet and subjected to severe heat stress increased milk protein content and improved economic returns, while reducing blood urea-nitrogen. Supplying additional RPM yielded no further production or economic benefits.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (P. Zhang).

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Competing Interest

The authors declare that they have no competing interests.

Author Contributions

P. Zhang designed the study. L. Li drafted and wrote the manuscript. H. Ling collected and analyzed the data. L. Li and J. Qu performed the animal trial and laboratory analysis. Q. Jiang, S. Tang and X. Lan revised the manuscript. All authors gave intellectual input to the study and approved the final version of the manuscript.

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Research Article

Evaluation of the Effects of New Combined Adsorbent on Occurrence of Pathological Lesions in Aflatoxin B1-Challenged Broiler Chickens

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Abstract: This experiment was performed to investigate the protective effect of a new combined adsorbent (bentonite + yeast cell wall extract) previously tested *in vitro,* on the occurrence of pathological lesions in broilers fed a diet contaminated with aflatoxin B1 until 21 days of age. A total of 96 one-day old Ross 308 hybrid broiler chickens were divided into four groups: group I (control), group II (5 g/kg adsorbent), group III (5 g/kg adsorbent + 2 mg/kg aflatoxin B1), and group IV (2 mg/kg aflatoxin B1). Grossly, AFB1 and AFB1+adsorbent fed birds showed enlargement, friable texture of liver and enlargement and pallorness of kidneys. Spleen revealed size reduction. No gross-pathological changes were observed in control and group II. Histopathologically, lesions were observed in aflatoxicated groups. Liver revealed vacuolar cell degeneration, periportal and perivascular infiltration of mononuclear cells. Kidneys revealed mild to moderate degree of haemorrhages, tubular epithelial necrosis and infiltration of mononuclear cells along with heterophils. In group IV, catarrhal enteritis characterized by desquamation of epithelial cells were found in the duodenum. A milder form of gross and histopathological lesions was seen in group III. In conclusion, the present study revealed that supplementation of a new combined toxin binder product in the concentration of 5 g/kg could not completely ameliorate aflatoxicity in broilers, although it met the stringent European regulation requirements for the minimum of 90% aflatoxin binding efficiency in *in vitro* study.

Keywords: *Adsorbent, Aflatoxin B1, Broilers, Pathological lesions*

Aflatoksin B1 İle Enfekte Edilen Etlik Piliçlerde Yeni Kombine Adsorbanın Patolojik Lezyonların Oluşumuna Etkilerinin Değerlendirilmesi

Öz: Bu çalışma, daha önce *in vitro* olarak test edilen yeni bir birleşik adsorbanın (bentonit + maya hücre duvarı ekstraktı), 21 günlük yaşa kadar aflatoksin B1 ile kontamine edilmiş bir diyetle beslenen etlik piliçlerde patolojik lezyonların oluşumu üzerine koruyucu etkisinin araştırılması için yapıldı. Toplam 96 adet bir günlük Ross 308 hibrit etlik piliç, grup I (kontrol), grup II (5 g/kg adsorban), grup III (5 g/kg adsorban + 2 mg/kg aflatoksin B1) ve grup IV (2 mg/kg aflatoksin B1) olmak üzere dört gruba ayrıldı. Büyük ölçüde, AFB1 ve AFB1+adsorban verilmiş piliçlerde karaciğerde büyüme ve gevrek bir doku ve böbreklerde büyüme ve solgunluk görüldü. Dalağın boyutunda küçülme saptandı. Kontrol ve grup II'de açık patolojik değişiklikler gözlenmedi. Aflatoksin verilen gruplarda histopatolojik lezyonlar izlendi. Karaciğerde vakuolar hücre dejenerasyonu, mononükleer hücrelerin periportal ve perivasküler infiltrasyonu görüldü. Böbreklerde, hafif ile orta derecede kanamalar, tübüler epitel nekrozu ve heterofillerle birlikte mononükleer hücre infiltrasyonu saptandı. Grup IV'te duodenumda epitel hücrelerde deskuamasyon ile karakterize kataral enterit saptandı. Grup III'te daha hafif açık ve histopatolojik lezyonlar görüldü. Sonuç olarak, bu *in vitro* çalışma, 5 g/kg konsantreli yeni bir kombine toksin bağlayıcı ürünün eklenmesinin, etlik piliçlerde aflatoksikozisi tamamen iyileştirmediğini, ancak Avrupa'daki minimum %90 aflatoksin bağlama etkinliği için katı düzenleme şartlarını karşıladığını ortaya koydu.

Anahtar sözcükler: *Adsorban, Aflatoksin B1, Broiler, Patolojik lezyonlar*

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Introduction

Mycotoxins represent toxic secondary metabolites of a wide range of fungi (principally molds) and have adverse effects on human and animal health. They cause a variety of acute or chronic diseases, collectively called "mycotoxicoses". There are over 400 identified mycotoxins [1] while aflatoxins, zearalenone, ochratoxin A, fumonisins, trichothecenes (such as deoxynivalenol - DON), and T-2 toxin are some of the mycotoxins that can significantly impact health and productivity of farm animals, including poultry species [2].

Since their first identification in the early 1960s, aflatoxins (AF) have been the most studied class of mycotoxins. The cause of aflatoxicosis in poultry and other food-producing animals has been attributed to the ingestion of various feeds contaminated with *Aspergillus* spp., and among the six types of aflatoxins, aflatoxin B1 (AFB1) is considered as the most potent and extremely toxic metabolite of this fungus [3]. Besides that, aflatoxin B1 is the most common metabolite in feeds and foods [4].

Aflatoxins cause a variety of adverse effects on the poultry health, including poor growth performance, disturbed gastrointestinal tract, immunosuppression, decreased weight gain, poor feed efficiency, reduced egg production and egg weight, changes in organ weights, and as well as carcinogenic, mutagenic and teratogenic effects [2,4-6]. The clinical signs and pathological changes caused by AFB1 in poultry have been widely described [2,7,8]. AFB1 causes a series of pathological changes in the organism and common pathological findings include the changes in the liver such as hepatomegaly, paleness, hydropic degeneration, fatty change, bile-duct hyperplasia and periportal fibrosis, kidney and spleen lesions and lesions in immune organs [9]. Sometimes there are no visible clinical signs and post-mortem lesions of mycotoxin intoxication, since in most cases, mycotoxicosis is chronic and caused by low-level ingestion of fungal metabolites, resulting in a decline in performance and the occurrence of nonspecific changes [2]. Usually, farm animals exhibit symptoms of chronic mycotoxicoses when exposed to feed contaminated with toxins below the guide-line levels [10].

Although there are a lot of innovative strategies for the reduction of mycotoxins in feed [11], one of the most common approaches to their detoxification includes the use of diverse mycotoxin binders [12]. The role of these inert adsorbents is to bind and immobilize mycotoxins in the gastrointestinal tract of animals, thus reducing their bioavailability and distribution to blood and target organs [13]. Since many of the studied adsorbents are expensive and may not be economically accessible for small farmers in developing countries, it is therefore important to investigate the use of locally available adsorbents for poultry feed decontamination [14].

Although Serbian national legislation regarding mycotoxins is harmonized with European legislation, only a small number of systematic monitoring programs are implemented and this cannot provide precise data about the occurrence of mycotoxins in feed in Serbia [15]. However, according to some previous studies, the presence of AF in corn from Serbia showed high contamination levels in corn and feed [16,17], and based on these results, it is known that AFB1 is frequently found in the corn originating from the province of Vojvodina, the north of Serbia.

From the point of view of diagnostic interest, the objectives of this study were to evaluate the gross and patho-histological lesions in different organs of broilers fed the diet that was experimentally contaminated with 2 mg/kg AFB1 and to determine the effectiveness of the new combined adsorbent on the appearance of pathological lesions in target organs. The other aim of this study was also a comparison of *in vitro* preliminary tests [18] of adsorption efficiency of new mineral adsorbent and an *in vivo* broiler trial.

Material and Methods

Experimental Design and Birds

The experiment was performed at the Institute for Animal Husbandry (Belgrade-Zemun). It was approved by Institute's Ethical Committee (Decision no. 323-07-03195/2020-05) and performed in accordance with the recommendations of the European Commission Directive 2010/63 EEC [19] and the Serbian Law on Animal Welfare [20]. A total of 96 one-day-old unsexed broilers (Ross 308 hybrid) were obtained from a local commercial hatchery. The birds were maintained in pens and divided into 4 equal groups - 24 per group, with 4 replicates of 6 birds each. The pen was the experimental unit. The chickens were exposed to 24 h of light every day. The temperature inside the experimental facility was initially 32±1°C and was gradually reduced by 3°C per week. Water and feed were given *ad libitum*. The chicks were inspected daily and any health-related problems were recorded. No vaccinations were administered during the experiment period.

Feeding Trial

Crystalline aflatoxin B1 with 99% purity used in this experiment was produced by Acros Organics™ (Geel, Belgium). Crystalline aflatoxin B1 was mixed with 1 kg ground corn using drum mixer RRM mini II (J. Engelsmann AG) for 5 min. Aflatoxin mixed ground corn was added to a basal diet to provide the required amount of 2 mg/kg feed. The used novel combined experimental adsorbent consisted of bentonite (smectite - dioctahedral montmorillonite) with yeast cell wall extract. This adsorbent was selected based on the adsorption characteristics obtained in *in vitro* studies [18]. Mycotoxin adsorbent was incorporated into the basal diet using counter-current

horizontal mixers. The experiment lasted for 21 days, and the broilers were fed a starter (from 1 to 14 days of age) and a grower (from 15 to 21 days of age). The chicks were randomly divided into four groups and each bird was weighed once a week. Group 1 (GI) served as a control group; group 2 (GII) received feed containing 5 g adsorbent/kg each day; group 3 (GIII) received 5 g adsorbent/kg + 2 mg AFB1/kg each day; group 4 (GIV) received feed containing 2 mg AFB1/kg, also for 21 days. The starter and grower feed for the experimental birds were tested for any possible residual aflatoxins and other mycotoxins, as described by Serbian legislation [21].

Gross Pathology and Histopathological Analysis

On the 21st day of the experiment, all the birds from groups were euthanized using carbon dioxide and then dissected. All the animals were weighed before euthanasia. Detailed necropsies were conducted immediately after killing the birds and gross lesions were reported. Additionally, for histopathological studies, tissue samples of the liver, kidneys, bursa of Fabricius, and duodenum were removed and collected in 10% neutral buffered formalin for 72 h. After fixation, the samples were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Five µm thick tissue sections were cut with a microtome and mounted on a glass slide. The slides were stained with hematoxylin and eosin (H&E) and examined under an Olympus light microscope (Olympus, BX51 Tokyo, Japan) for histopathological changes.

Histomorphometric Analysis

Histomorphometric analyses were done to evaluate the height of the duodenal villi. The same segment of the duodenum from each bird in the group was sampled (approximately 1 cm segment from the middle part of the duodenum was dissected). Using the light microscopy 10 x objective lens, ten well-oriented villi from each duodenum were randomly selected. The measurements were performed using the Olympus BX51 microscope with a digital CCD camera (Color View III, Olympus) connected to a computerized image analysis system (Olympus Cell B, Olympus, Japan). The length of the duodenal villi (expressed in µm) was measured from the villus-crypt junction to the top of the villi.

Statistical Analysis

Statistical analysis was performed using R version 3.2.2 statistical software (R Foundation for Statistical Computing, Vienna, Austria). The data were analyzed using one-way models of analysis of variance (ANOVA). Significant differences between the groups were set at $P \leq 0.05$ and probabilities were determined by Duncan's post hoc test.

Results

Gross Lesions

No mortalities occurred during the experiment period and in general, all the birds were in good condition. The birds belonging to Group I (Control) had a normal morphological appearance of all the organs throughout the experimental study. In the birds from Group II (GII), the only detected macroscopic lesion was the hypertrophy of bursa Fabricius in 2 chickens. Livers were normal in color, size and consistency in all chicks from groups GI and GII. The constant gross lesions in the intoxicated birds from groups III and IV were atrophy of the spleen and hypertrophy of bursa Fabricius. The birds from group III showed moderate enlargement and congestion of the liver and moderate enlargement of kidneys. The most striking macroscopic lesions were in aflatoxin-fed group IV (GIV). The majority of birds had enlarged and edematous bursa Fabricius *(Fig. 1-A)*, enlarged kidneys and livers. The liver was congested, swollen and friable and occasionally small yellowish foci were seen. Enlarged kidneys were mostly pale *(Fig. 1-B)*, and the presence of petechial and ecchymotic hemorrhages was detected. Mild hemorrhages were occasionally present in the mucosa of the small intestines. The heart and subcutaneous tissues were normal in appearance and no other macroscopic lesions

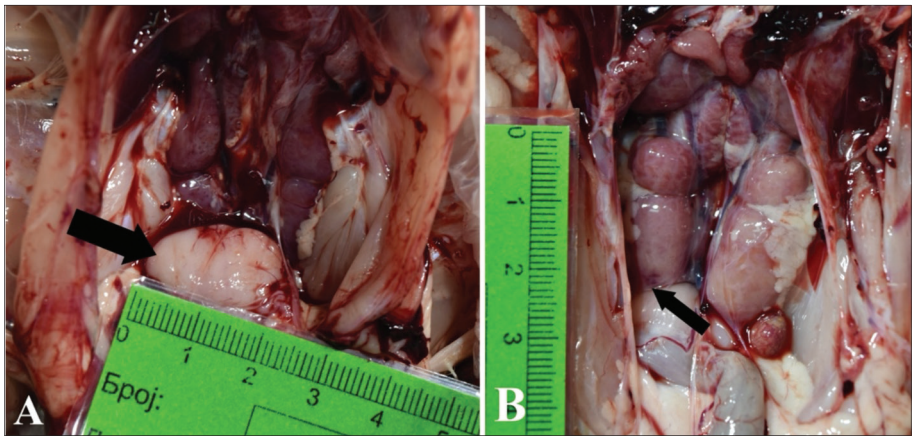


Fig 1. Gross lesions in aflatoxicated broiler chickens (Group IV). A- Enlarged and swollen bursa Fabricius; B- Enlarged and pale kidneys followed by petechial haemorrhages

Table 1. *Effects of combined adsorbent (bentonite + yeast cell wall extract) and AFB1 on gross lesions of broiler chickens at 21st day of age*

Lesions		Groups			
		Group I	Group II	Group III	Group IV
Enlargement of organs	Liver	-	-	5/24	9/24
	Kidney	-	-	8/24	15/24
	Bursa Fabricius	-	2/24	5/24	13/24
Hemorrhages	Liver	-	-	3/24	7/24
	Kidney	-	-	3/24	6/24
	Intestine	-	-	3/24	8/24
	Spleen	-	2/24	5/24	7/24
Friable liver		-	-	5/24	8/24
Pallor kidneys		-	-	8/24	12/24
Spleen atrophy		-	-	5/24	9/24

Legend: no change/lesion (-); The values represent the birds showing gross lesions/number of birds examined in each experimental group

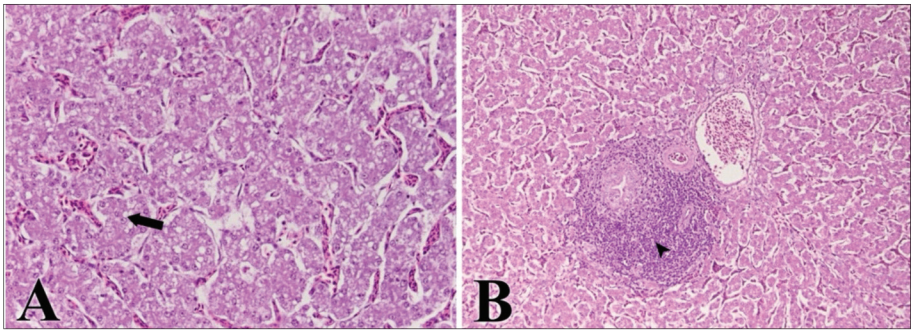
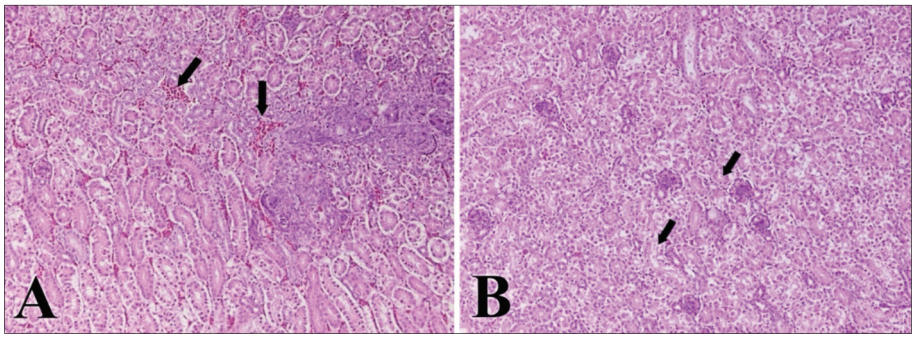


Fig 2. Liver of broiler (Group IV) fed with 2 mg AFB1/kg, 21st day of intoxication. A- Degeneration of hepatocytes having vacuolated cytoplasm *(arrow)*, HE x 400; B- Pericholangitis *(arrowhead)*, HE x 200

Fig 3. Kidney of broiler (Group IV) fed with 2 mg AFB1/kg, 21st day of intoxication. A- Heamorhhages *(arrows)*, HE x 200; B- Necrosis in renal tubular epithelium *(arrows)*, HE x 200



were detected in all the examined birds. The detected gross lesions and their frequency in birds are in *Table 1*.

Histopathological Lesions

The birds from groups I and II showed no histopathological alterations and normal anatomical structure was present in all birds. In the birds belonging to groups III and IV, microscopic lesions of the liver consisted of hepatic vacuolar cell degeneration *(Fig. 2-A)* and accumulation of a large number of heterophils and multifocal mono nuclear cell infiltration in the parenchyma, as well as pericholangitis *(Fig. 2-B)*. Less frequent lesions of the liver were perivascular infiltration and moderate to severe multifocal hemorrhages and multifocal necrotic foci. Another microscopic lesion observed in the liver of all birds from both aflatoxicated groups was congestion of the hepatic sinusoids. Comparing both groups, the described microscopic lesions in the liver were less severe in group III. Moderate degree of sinusoidal congestion was revealed in the kidneys of aflatoxin fed birds, as well as mild to moderate focal hemorrhages *(Fig. 3-A)*, mild to moderate tubular epithelial necrosis *(Fig. 3-B)*, with the infiltration of aggregates of mononuclear cells along with heterophils. The kidneys of birds from group III revealed mild degree of congestion and mild degree of tubular epithelial necrosis. The spleens were hyperemic, and reactive hyperplasia of secondary follicles was revealed, resulting in the widening of a lymphoid component. In group IV, catarrhal enteritis characterized by desquamation of epithelial cells was found in the duodenum. No lesions were observed in bursa

Table 2. Histomorphometric values of duodenal villi length of broiler chickens on the 21st day of experiment

Group	Length of Duodenal Villi (µm)
Group I	974.11±162.21[a]
Group II	972.85±155.06[a]
Group III	981.49±145.68[a]
Group IV	819.32±193.29[b]

Means within the same column with no common superscript letter are significantly different (P<0.05). Data are means with standard deviation

of Fabricius that could be interpreted as a characteristic of the process.

Histomorphometric Observation

Histomorphometric observations are presented in *Table 2*. There was a significant difference (P<0.05) in the length of duodenal villi between group IV and other treatment groups and control group. There was no significant difference between treatment groups (group II and III) and control group.

Discussion

The majority of the research is focused on the de-contamination and detoxification of feed from aflatoxin B1, since their frequent occurrence in feedstuffs cause severe health problems in the poultry industry and large economic losses. *In vitro* preliminary tests of mycotoxin adsorbents are considered as a powerful tool for screening the potential of mycotoxin-detoxifying agents since if no adsorption occurs *in vitro*, there is little or no chance to do so *in vivo* [22]. In this study, the ability of a new combined mycotoxin adsorbent to detoxify poultry feeds contaminated with aflatoxin B1 was tested in *in vivo* broiler trial. The absence of pathological lesions in birds from the control group and birds fed on adsorbent alone indicate that the new combined adsorbent is non-toxic and inert. Similar observations were made by Manafi et al.[23], Lakkawar et al.[24] and Ortatatli and Oguz [25] who reported that the chicks after receiving binders alone did not have any pathological changes. The harmful consequences of AFB1 administration on the pathological lesions of chickens are widely reported, and many studies showed that naturally-contaminated diets cause the most harm to the birds [26]. The adverse effects reported earlier have also been identified in the experimental feeding of AFB1 in the present study.

In poultry, aflatoxin B1 mostly affects the liver, kidney, immune organs (spleen, bursa of Fabricius and thymus) and gastrointestinal system. Considering that liver is a major metabolizing and detoxifying organ in the body, the exposure to AFB1 leads to liver damage in different animal species [27] causing various pathological changes such as enlargement, congestion, necrosis, pallor, discoloration and fatty liver syndrome [9,28]. Prolonged exposure to aflatoxins leads to the increase in the relative mass of the liver due to increased vacuolization of hepatocytes and accumulation of large amount of lipids. In this study, liver lesions were observed in aflatoxicated birds and birds fed AFB1 diet + adsorbent. The described liver lesions of birds from group III showed a milder degree of severity and enlargement, compared to mycotoxin treated birds. Contrary to previous findings [8,24,29], the livers examined in this study did not show any pallor or increased lipid content. The group which received adsorbent alone did not show any significant histopathological changes of the liver as was the case in control group. The main histopathological changes in the liver of birds from group III and IV included vacuolar degeneration and accumulation of lymphoid cells that is in agreement with those reported by Lakkawar et al.[24], Ahmed et al.[7], Denli et al.[30]. From previously discussed reports [7,23] as well as from our findings, it is clear that even short-term exposure to AFB1 has negative effects in broiler chickens and induce pathological changes in the liver.

Kidneys also take part in detoxification of aflatoxins, making them very vulnerable organs to AF. The kidneys are among the organs in which aflatoxin residues are most often detected [31], making them the target organs for AF. Various studies have shown that AFB1 leads to congestion of the renal sinusoids and an increase in the relative mass of the kidneys. Besides, AFB1 leads to degenerative and necrotic changes in the tubular epithelium, reduction of glomerular filtration thereby impairing renal function [9]. The most common macroscopic changes in birds treated with AFB1 are kidney enlargement with pallor [25,28,29] which is what our research has proved in the majority of the birds belonging to Group IV. Histopathological changes in the kidney of chickens exposed to AFB1 and AFB1 + adsorbent are comparable to those reported in the literature on avian aflatoxicosis. The birds from group III showed a milder degree of severity and enlargement of kidneys as compared to mycotoxin treated birds.

Since the gastrointestinal tract (GIT) is the major spot of conversion and absorption of food components, it is expected that the negative effects of mycotoxins will be manifested to a greater extent in the organs of the GIT. However, the available literature data on the effect of mycotoxins on GIT organs of poultry are scarce, and the results of the researches are controversial [32,33]. Such discrepancies can be explained by the fact that various poultry species were used in the studies, different parts of the GIT were examined as well as the doses of AFB1 and lengths of exposure varied [9]. In this study, pathohistological lesions of the intestine were detected only in the chickens exposed to AFB1. Catarrhal enteritis with epithelial desquamation was observed. Similar results were reported by Kumar and Balachandran [34]. Moreover,

our results prove that AFB1 in the quantity of 2 mg/kg leads to shortening of the duodenal villi, which is in agreement with the results of Yang et al.[35]. Likewise, the same findings in duodenum were reported in turkeys naturally intoxicated with deoxynivalenol [36]. On the other hand, some authors did not observe any significant histological lesions in intestinal epithelium in broiler chickens intoxicated with AFB1 [33,37]. Another group of authors has proved that the use of AFB1 in large dozes does not lead to histopathological changes of intestines [33,38]. Based on the literature available and aforementioned studies, the response of the gastrointestinal tract to the aflatoxin B1 is non-conclusive, and it is difficult to determine a dose-effect relationship between AFB1 and histological changes in the intestines of the chicken.

In various studies conducted in the past decades, it has been shown that AFB1 decrease immune responses [28,39], and as a result of AFB1-induced immunosuppression, the exposed poultry have lower resistance to secondary infections. AFB1 in poultry can cause damage on primary and secondary lymphoid organs including thymus, bursa of Fabricius, spleen, and bone marrow [9]. Absolute and relative weights of immune organs are used to indirectly assess the immune status of birds, so the changes in their relative weights may result in altered immune function [40]. In our study, the reduction in the size of the spleen was recorded in a majority of birds of both aflatoxicated groups. Previously it has been reported [41] that reduced relative weight of the spleen caused by AFB1 may occur because its white pulp contains less lymphoid tissue. However, in this study, microscopical findings showed widening of the lymphoid component along with the formation of secondary lymphoid follicles, which is in agreement with Lakkawar et al.[24]. The bursa Fabricius was enlarged in aflatoxicated birds, which is in agreement with Pandey and Chauha [29].

Even though preliminary *in vitro* tests showed a high level of binding capacities of new combined adsorbent, *in vivo* results showed its partial success. The addition of 5 g/kg adsorbent to the diet of birds supplemented with 2 mg/kg AFB1 partially compensated the harmful effects of the aflatoxin B1 on the liver, spleen and kidney and also partially decreased the incidence of affected broilers. The duration of exposure to AFB1 and adsorbent could be more effective with an extension of the experiment that would probably cause more distinct morphological changes in target organs. Differences in *in vivo* efficacy compared to the expectations based on *in vitro* testing, indicate that *in vitro* results alone are not adequate for the evaluation of adsorbents. Comprehensively concluding, it is clear that examination of additives to lessen the impact of mycotoxins is a time-consuming and complex process that requires maximum commitment and repeated attempts to reach the best solution.

Availability of Data and Materials

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

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Competing Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

BDj, MP, SK, VP, KN conceived and planned the study design. BDj drafted the manuscript. BDj, MP and SK carried out the experiment, performed necropsy, examined and described gross lesions and made substantial contribution to collection, analysis and interpretation of the data. IV worked out in histopathological analysis and made interpretation of the results. SK verified statistical analysis and made substantial contributions to interpretation of data. VP, KN and DOA made contributions to conception and design of the article, and carried out literature research. VP, MP, KN, IV and DOA read the manuscript and made corrections in the document. All authors have approved the final version of the manuscript.

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RESEARCH ARTICLE

Effects of Melatonin Addition to the Cold Storage Medium on Cumulus Oocyte Complex Apoptosis, Viability and *In Vitro* Maturation Rates of Cat Oocytes

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Abstract: Usage of oocytes obtained from ovaries after long-term cold storage for *in vitro* embryo production is a promising tool for the protection of wildlife and endangered animal species. Mammalian oocytes are susceptible to oxidative stress with regard to the high lipid content of plasma membranes. Melatonin is known as a powerful antioxidant and anti-apoptotic agent due to its ability to eliminate toxic oxygen derivatives and reduce the formation of reactive species. This study was performed to verify the optimal environmental conditions for long-term preservation of cat ovaries *(Felis domesticus)* by adding different concentrations of melatonin (500, 750 and 1000 µM) to the storage medium (0.9% NaCl) as an antioxidant to be preserved at 4°C for 24 h. To determine the effect of melatonin on cat oocytes collected from stored ovaries, the anti and pro-apoptotic gene levels in cumulus oophorus, the *in vitro* maturation rates, the cell membrane and oocytes viability were evaluated. In all melatonin added groups regardless of whether they are stored in the cold; Pro-apoptotic gene levels (BAX) were determined to be upregulated however, anti-apoptotic gene levels (BCL-2) were downregulated in cumulus cells ($P<0.05$). The cell membrane stability and cell viability rates of oocytes began to deteriorate in parallel with the rate of melatonin increase. In parallel with these findings, *in vitro* maturation rates of oocytes were negatively affected as the amount of melatonin increased ($P\leq0.001$). In conclusion the results showed that adding melatonin (500,750 or 1000 µM) to the ovarian transport and storage medium had negative effect on *in vitro* maturation rate, viability and cell membrane structure of cat oocytes.

Keywords: *Cold storage, Melatonin, Cat ovaries, In vitro maturation, Apoptosis, Bax, Bcl-2*

Kedi Ovaryumlarının Soğukta Saklama Solüsyonuna Melatonin Katılmasının, Kümülüs Ooforuslardaki Apoptoz ve Oositlerin Viyabilitesi ve *İn Vitro* Olgunlaşma Oranlarına Etkisi

Öz: *İn vitro* embriyo üretimi amacıyla, ovaryumların soğuk ortamlarda uzun süreli saklanması sonrası elde edilen oositlerin kullanılabilmesi, yaban hayatı ve nesli tükenmekte olan türlerin korunmasına yardımcı olabilir. Memeli gametleri, plazma zarlarındaki yüksek lipid içeriği nedeniyle, oksidatif strese karşı savunmasızdırlar. Melatonin, toksik oksijen türevlerini ortadan kaldırma ve reaktif türlerin oluşumunu azaltma özelliğinden dolayı güçlü bir antioksidan ve anti-apoptotik ajan olarak bilinmektedir. Çalışma, 4°C'de 24 saat süreyle muhafaza edilen ovaryum taşıma sıvısına (%0.9 NaCl) antioksidan olarak farklı dozlarda melatonin (500, 750 ve 1000 µM) eklenerek evcil kedi ovaryumlarının uzun süreli muhafazası için en uygun koşulları belirlemek amacıyla yapılmıştır. Melatoninin soğukta bekletilmiş ovaryumlardan kazanılan kedi oositleri üzerindeki etkisini belirlemek amacıyla; kümülüs ooforuslardaki anti ve proapoptotik gen seviyeleri, oositlerdeki *in vitro* olgunlaşma oranları, hücre membran stabilitesi ve hücre canlılığı gibi parametreler incelendi. Soğukta bekletilsin ya da bekletilmesin, tüm melatonin ilaveli gruplarda; kümülüs hücrelerindeki pro-apoptotik gen seviyelerinin (Bax) yükseldiği, buna karşın anti-apoptotik gen seviyelerinin ise (Bcl-2) düştüğü gözlendi ($P<0.05$). Oositlerin hücre membran stabilitesi ve hücre canlılık oranları da melatonin artışına paralel olarak bozulmaya başladığı görüldü. Bu bulgulara paralel olarak, melatonin miktarı arttıkça oositlerin *in vitro* olgunlaşma oranları da olumsuz yönde etkilendi ($P\leq0.001$). Sonuç olarak, yumurtalık saklama sıvısına 500, 750 ve 1000 µM dozlarında melatonin eklenmesinin, kümülüs hücrelerinde apoptozu teşvik ettiği, kedi oositlerinin *in vitro* olgunlaşma oranları, hücrelerin canlılığı ve hücre membran yapısı üzerinde de olumsuz etkileri olduğu sonucuna varıldı.

Anahtar sözcükler: *Soğukta saklama, Melatonin, Kedi ovaryumları, İn vitro maturasyon, Apoptozis, Bax, Bcl-2*

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Introduction

Most of the wild cat species are facing extinction due to loss of habitat and poaching. Assisted reproductive technologies such as *in vitro* maturation, *in vitro* fertilization and nuclear transfer offers a tremendous potential for conservation of endangered cat species [1]. Domestic cat is not only a convenient model for developing assisted reproduction of endangered felids but also for researching human genetic diseases [2,3]. Many inherited genetic disorders have been identified in cats that resemble humans. In recent years cat model has become widely used for therapeutic studies in human [2-4].

Prolonged preservation of the ovaries under optimal conditions may allow producing new generations from endangered wild felids that undergo ovariectomy or have recently died in their habitat or zoo [5]. However, long-term transport of the ovaries under warm temperatures (35-38°C) has negative effects on oocyte quality in terms of *in vitro* maturation, fertilization and subsequent embryonic development [6]. A number of studies have been conducted to increase oocyte protection by reducing temperatures of transport solution [1,5,7-9]. However, the sensitivity of oocytes to transport conditions was found to vary among animal species [1,5]. Cumulus cells surround the oocyte, keep the oocyte under meiotic arrest, exchange of nutrients and regulate chemical signals related to oocyte development and functions in cytoplasmic maturation of oocytes [10-12]. The viability of the oocyte and cumulus oophorus cells is effective on the cytoplasmic maturation of the oocyte which is crucial for the growth potential of embryos after fertilization [10]. Transport temperature and time may affect the metabolism and thus apoptosis of cumulus cells and *in vitro* maturation rates of cat oocytes [5]. Reactive oxygen species (ROS) have also been shown to cause DNA damage in human embryos and thus induce apoptosis *in vitro* [13]. Apoptosis, also known as programmed cell death, is a physiological process that is defined by the self-destruction of cells and this process is controlled by genes. Reactive oxygen species (ROS) which are known to have mutagenic effects on DNA are produced as metabolites by normal cellular processes. Although all cells own self defense systems containing antioxidant enzymes, their levels are not the same in all cell types [14]. Mammalian gametes are extremely susceptible to oxidative stress due to their high lipid content. Melatonin (N-acetyl-5-methoxtryptamine), which is classified as an indoleamine is a tryptophan derivative synthesized in the pineal gland and regulates seasonal reproductive ability. Melatonin is also known as a direct and indirect antioxidant and anti-apoptotic agent that prevents from oxidative damage due to its ability to scavenge toxic oxygen derivatives and reduce the formation of ROS [15]. Melatonin neutralizes potentially toxic molecules, particularly single oxygen, hydrogen peroxide, nitric oxide and peroxynitrite anion and stimulates several antioxidative enzymes. In the mammalian ovary, as in other organs, both membrane and nuclear receptors for melatonin have been identified [16,17]. Melatonin has been shown to have beneficial effects on stimulating oocyte maturation, embryo production and blastocyst rates *in vitro* in some mammalian species [7-9].

The most effective method for assessing oocyte maturation conditions *in vitro* is the evaluation of the mRNA expression of specific genes related to apoptosis in granulosa cells [18]. BCL-2 is a member of the BCL-2 regulatory family of proteins that regulate cell apoptosis.

BCL-2 is a noteworthy anti-apoptotic gene that inhibits programmed cell death by reducing the formation of ROS, thereby preventing intracellular oxidations necessary for induction of apoptosis. BAX is a member of the BCL-2 protein family and is a pro-apoptotic gene. BAX stimulates apoptosis through interaction with anion channels [19] and the ratio of pro- and anti-apoptotic genes indicates whether a cell undergoes apoptosis [20].

This study proposes the hypothesis that using melatonin in storage media of cat ovaries may have beneficial effects by preventing apoptosis during long-term protection under cold conditions. Therefore, the influence of melatonin addition to the ovary storage medium on COC's apoptosis, oocyte developmental rate and cell membrane viability of domestic cat oocytes were investigated.

Material and Methods

All chemicals were acquired from Sigma Chemical Co. (Saint Louis, MO, USA) except otherwise indicated. Melatonin (M-5250) was dissolved in absolute ethanol before adding to PBS due to its low solubility of in water.

Experimental Design, Collection - Storing of Ovaries and Oocyte Recovery

The ovaries which are obtained from routine cat spay surgeries of 60 cats in total at Istanbul Metropolitan Municipality Animal Sterilization Centre were transported in saline solution (0.9% NaCl) in flask at 4°C and brought to the laboratory within maximum two hours. All of the cats were stray cats, and the cycle period they are in is not taken into account.

The study was performed in two stages. In the first stage of the study, transported ovaries in saline solution added melatonin at different concentrations (500, 750 and 1000 µM) were divided into two parts. Half of ovaries were sliced immediately for the collection of COCs (Fresh Groups) and the others were stored under 4°C for 24 h (Stored Groups). Slicing was done as we did in our previous studies [5]. Briefly, ovarian cortex was gently cut with a scalpel and then washed with warm oocyte washing

medium on watch glass. The COCs with at least 4 rows of cumulus oophorus cell lines, an intact zona pellucida and dark ooplasm were selected. Some of the collected COCs were separated to determine the expression of possible pro-apoptotic (BAX) and anti-apoptotic (BCL-2) genes and some of oocytes were separated and stained for assessment of cell membrane damage and oocyte cell viability and the remaining oocytes were matured *in vitro*.

At the second stage of the study, the stored ovaries at 4°C for 24 h with different melatonin concentrations (0, 500, 750 and 1000 µM) were also sliced and the COCs were collected. Some COCs were separated to determine the expression levels of BAX and BCL-2 genes and several oocytes were separated and stained for assessment of cell membrane damage and oocyte cell viability. Then the others were left for *in vitro* maturation. After IVM period, several COCs were also separated from each group to analyze the expression levels of the studied genes in cumulus cells and the rest oocytes were examined.

In Vitro Maturation

In vitro maturation was performed as described in our group's previous work [5]. Briefly, the maturation medium was modified Synthetic Oviduct Fluid (SOF), supplemented with 10 µg/mL recombinant follicle stimulating hormone (rFSH), 10 µg/mL recombinant luteinizing hormone (rLH), 4% bovine serum albumin (BSA, Fraction V) and antibiotics. The selected COCs were maturated at 38°C for 48 h in four-well petri dishes (NUNC, Denmark) including 500 µL maturation medium covered with mineral oil. A humidified atmosphere at 38°C with 5% CO_2 was used for 48 h for the *in vitro* maturation process *(Table 1, Table 2)*.

Assessment of the Nuclear Maturation

After the 48 h of IVM period, oocytes were denuded by vortexing for 60-90 sec after being transferred into hSOF medium containing 0.2% (w/v) hyaluronidase and pipetting to remove the remaining cumulus cells. The oocytes were evaluated under epifluorescence microscope (IX 70, Olympus, Japan) equipped with a digital camera after 20 min 1 mg/mL Hoechst 33342 staining.

Assessment of Cell Membrane Damage, Oocyte Cell Viability and Nuclear Maturation

The oocytes separated from cumulus cells were stained with the Annexin V-FITC Early Apoptosis Detection Kit (6592, Cell Signal Technology) following the manufacturer's instructions. During the whole process, the reagents were kept at 37°C to avoid false positives. Cells were stained with Annexin-V, a phospholipid binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane, which takes place during the early stages of apoptosis. Cells were also stained with propidium iodide (PI), a membrane impermeable stain, to distinguish between live cells and dead cells. PI can only penetrate into the cell if cytoplasmic membrane has lost its integrity. Samples were placed in 35 µL droplets containing Annexin-V buffer, Annexin-V/FITC, PI and 1 mg/mL Hoechst 33342, and incubated for 15 min at 37°C in the dark. After the incubation oocytes were fixed for 10 min using 1% Paraformaldehyde (PFA) on ice. After fixation, the oocytes were washed three times in annexin-binding buffer and then mounted on glass slides which were examined using an (IX 70, Olympus, Japan) epifluorescence microscope equipped with a digital camera.

Denuded oocytes were classified as follows:

I. Live early apoptotic oocytes: Annexin-positive signal on the membrane (more than 20%) (A+/PI–) *(Fig. 1-A)*

II. Necrotic oocytes: PI-positive red nuclei, or cytoplasm (P+) *(Fig. 1-B,D)*

III. Live non-apoptotic oocytes: no annexin staining (A–/PI–) *(Fig. 1-C)*

During the evaluation of the oocytes, the advancement of nuclear maturation was also recorded *(Table 3)*.

Gene Expression Analysis

The differences in the expression of the studied genes related to apoptosis mechanism namely, B-cell lymphoma protein 2 (BCL-2) and Bcl-2-associated X protein (BAX) within the studied groups were analyzed with quantitative real time PCR (qRT-PCR) method using LightCycler 480

Table 1. *The in vitro maturation rates of oocytes recovered from transported ovaries in 0.9%NaCl supplemented with various concentrations of melatonin at 4°C (Fresh Groups)*

Melatonin (µM)	No. of Oocytes Examined (n)	Developmental Stage of Oocytes					
		GV (%)	GVBD (%)	MI (%)	MII (%)	UDNM (%)	Activated (%)
0	307	14.0	16.6^{b}	13.4	48.2^{ab}	6.5^{a}	1.3
500	436	11.5	25.7^{a}	12.6	45.0^{bc}	3.4^{b}	1.8
750	432	15.5	23.8^{a}	16.0	40.3^{c}	3.0^{bc}	1.4
1000	403	11.2	27.5^{a}	15.4	39.5^{c}	4.5^{ab}	2.0

Different superscripts within the same column indicate significant differences (P< 0.05)
GV: *Germinal Vesicle* ***GVBD:*** *Germinal Vesicle Break Down* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

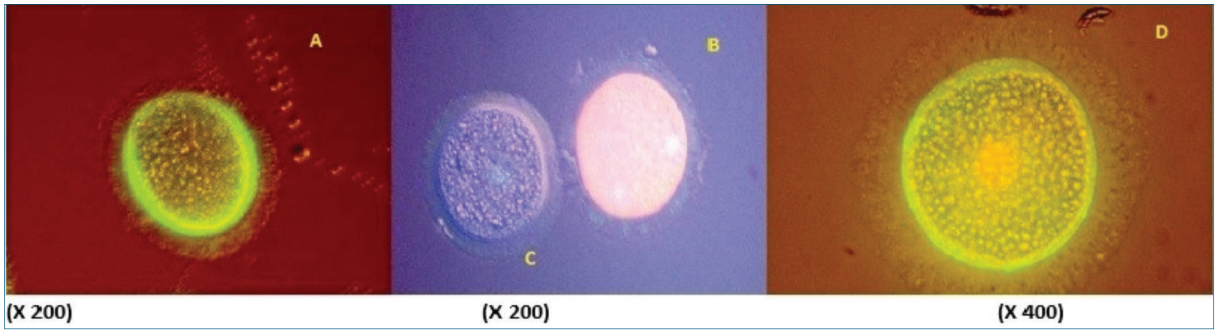

Fig 1. Cat oocytes stained with Annexine V and Propidium Iodide (PI) visualized under an epifluorescence microscope. **A**- An oocyte with membrane defect. Annexine (+) (green fluorescent more than 20%), **B**- A necrotic oocyte. PI (+) (red fluorescent), **C**- A normal oocyte, with healthy membrane and oolplasm, **D**- An oocyte necrotic and with membrane defect (PI+, Annexine+)

Equipment (Roche Applied Science). In this procedure we used a commercial kit (BioRad, SingleShot SYBR Green kit) that enabled the direct use of cell lysates bypassing the RNA isolation step. Briefly, *in vitro* matured cumulus-corona samples were evaluated for the cell count under a light microscope. Dilutions of 10^5 cells were washed in nuclease-free PBS (phosphate buffer solution) and cell lysates prepared in cell lysis buffer (SingleShot Cell Lysis Buffer, BioRad) were snap frozen and stored at -80°C until genetic analyses. For cDNA synthesis, 4 µL of cell lysate was added to reverse transcription mixture (iScript Advanced Reaction Mix, BioRad). Six replicates were done for each group.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The Felis catus mRNA-specific primers for BCL-2 (NM_001009340.1), BAX (NM_001009282.2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_001009307.1) were designed using Primer3 and checked using Oligo-analyzer 3.1 tool *(http://www.idtdna.com)*. The sequence homology was confirmed using the BLAST database *(http://blast.ncbi.nlm.nih.gov/Blast.cgi)*. Primer sequences were 5'-AGTACCTGAACCGGCACCT-3' and 5'-GGACAGC CAGGAGAAATCAA-3' for BCL-2, 5'-TCAAGGCCCT GTGTACCAA-3' and 5'-TGGGTGTCCCAAAGTAGG-3' for BAX, 5'- CAATGACCCCTTCATTGACC-3' and 5'-ATG GGCTTTCCATTGATGAC-3' for GAPDH. qRT-PCR was performed using LightCycler 480 (Roche Applied Science) equipment using SsoAdvanced Universal SYBR Green Super Mix (BioRad). The cumulus cell expression levels of BCL-2 and BAX genes were normalized with GAPDH [21]. The results were expressed as relative quantification (RQ) values and calculated with comparative 2–ΔΔCt method. All samples were studied in triplicates. The specificity of the amplification products was confirmed by melting curve analysis *(Table 4)*.

Statistical Analysis

The statistical analysis was implemented using a purpose-built software (IBM, SPSS Version 14.0). The rates of apoptotic oocytes and nuclear maturation were analyzed by Chi-square test and the significance level was set at $P<0.05$. The comparisons in BCL-2 and BAX expressions (RQ values) in the study groups were evaluated by Mann-Whitney U test. Probabilities of <0.05 were deemed statistically significant.

Results

The outcomes of the study are presented in *Table 1, Table 2, Table 3* and *Table 4*. The meiotic status of oocytes recovered from transported ovaries in 0.9% NaCl supplemented with various concentrations of melatonin at 4°C summarized in *Table 1*. The MII stage oocyte rate was higher in control group than the melatonin trial groups, and this difference increased with accelerating melatonin concentrations ($P<0.001$). The meiotic status of oocytes recovered from ovaries kept at 4°C for 24 h are summarized in *Table 2*. The IVM rates in this groups were parallel to the Fresh groups result.

The apoptosis rate and viability status after IVM of oocytes recovered from ovaries whether kept at 4°C for 24 h or not are summarized in *Table 3*. The rate of apoptosis and dead oocyte rates were increased in parallel with the amount of melatonin added to the medium.

The effects of storing of ovaries for 24 h at 4°C in 0.9% NaCl supplemented with melatonin on BCL-2 and BAX gene expression results were summarized in *Table 4*. The oocytes' cell membrane and viability were affected negatively as the amount of melatonin increased.

After IVM, BCL-2 expression was downregulated in a dose dependent manner whereas BAX expression was not comparable between the groups. The alteration in expression of BCL-2 gene was more prominent with increasing doses of melatonin in both fresh and stored groups. On the other hand, BCL-2/BAX ratio, which is an indicator of apoptosis protection in cells, is significantly decreased with increasing melatonin concentrations ($P<0.05$) and this ratio is mainly influenced by the

Table 2. *The in vitro maturation rates of oocytes recovered from ovaries kept at 4°C for 24 h (Stored Groups)*

Groups	Melatonin (µM)	No. of Oocytes Examined (n)	Developmental Stage of Oocytes				
			GV (%)	GVBD (%)	MI (%)	MII (%)	UDNM (%)
Control Group *(Ovaries not stored)*	0	246	16.7	7.3^{b}	17.9	55.7^{a}	2.8
Trial/Storage Groups *(Ovaries kept at 4°C for 24 h with adding Melatonin)*	0	267	10.5	17.6^{a}	15.0	52.4^{a}	5.6
	500	384	10.9	21.9^{a}	13.8	47.7^{ab}	11.7
	750	376	15.7	21.3^{a}	17.0	41.8^{b}	8.8
	1000	350	12.3	23.4^{a}	15.7	41.4^{b}	13.4

Different superscripts within the same column indicate significant differences (P<0.05)
GV: *Germinal Vesicle,* ***GVBD:*** *Germinal Vesicle Break Down,* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

Table 3. *Apoptosis and viability status of in vitro maturated oocytes recovered from ovaries whether kept at 4°C for 24 h or not*

Groups	Melatonin (µM)	No. of Oocytes Examined (n)	Live Nonapoptotic Oocyte (A-PI-)(%)	Live Apoptotic Oocyte (A+- PI-) (%)	Dead Oocytes (P+) (%)
Fresh Group *(Ovaries not stored)*	0	246	84.1^{a}	2.4^{c}	13.4^{b}
Stored Groups *(Ovaries stored at 4°C for 24 h with adding Melatonin)*	0	267	80.5^{ab}	3.4^{c}	14.6^{b}
	500	384	76.6^{b}	8.3^{b}	14.3^{b}
	750	376	64.6^{c}	11.2^{ab}	23.1^{a}
	1000	350	63.4^{c}	13.1^{a}	23.1^{a}

Different superscripts within the same column indicate significant differences (P<0.05)

Table 4. *Effects of varies storage at 4°C for 24 h or not in melatonin supplemented 0.9% NaCl on BCL-2 and BAX gene expression results*

Groups		Gene Expression Results						BCL-2 /BAX
		BCL-2			BAX			
		Mean Value	Standard Dev.	P Value	Mean Value	Standard Dev.	P Value	
Trial Groups		0.10	0.04	0.86*/0.2**	0.28	0.01	0.38*/0.2**	10
Fresh Groups (Ovaries not stored at 4°C for 24 h with adding Melatonin)								
Amount of Melatonin (µM)	0	0.82	1.00	-	0.96	0.52	-	0.85
	500	0.48	0.90	0.73*	0.58	0.80	0.43*	0.83
	750	1.20	0.69	0.55*	1.31	0.77	0.31*	0.92
	1000	2.86	4.09	0.55*	1.79	1.58	0.69*	1.60
Stored Groups *(Ovaries stored at 4°C for 24 h with adding Melatonin)*								
Amountof Melatonin (µM)	0	0.69	0.51	1.00*	1.17	0.74	0.79*	0.59
	500	0.84	0.81	1.00**	0.92	0.69	0.86**	0.91
	750	0.57	0.53	0.86**	0.90	0.72	0.86**	0.63
	1000	0.34	0.19	0.40**	1.02	0.66	0.86**	0.33

The P value gives the Mann-Whitney U test results between the groups (P<0.05)
** Comparison the values between Fresh and Control Groups; ** Comparison the values between Stored and Control Groups*

expression changes in BCL-2 gene. Photographs of the necrotic, live, apoptotic and non-apoptotic oocytes are given in *Fig. 1*.

Discussion

During the transport of the ovaries to the laboratory, the interruption of blood flow cuts off the energy and puts the ovaries in an ischemic state. Therefore, ROS begin to accumulate in the follicle microenvironment. ROS react with proteins, lipids and DNA of oocyte and cumulus cells, causing cell membrane-lipid peroxidation, DNA damage and ultimately apoptosis and for an effective embryo production program, oocyte oxidative stress caused by ROS should be limited [8,17]. The prolonged time during the transport of the ovaries can cause apoptosis in the cells by disrupting the enzymatic activities that play a role in oocyte maturation. ROS which causes apoptosis in oocytes have also been shown to cause DNA damage in porcine and human oocytes during in vitro culturing [13]. Although it is suggested that storage of cat ovaries at 4°C for 12 h initiates DNA degradation in granulosa cells [22], some researchers stated that cat oocytes have a unique ability regarding to *in vitro* maturation after ovary storage 24 h at 4°C [23]. Our previous study results [1] also showed that storing of domestic cat ovaries in saline up to 24 h at 4°C did not have a negative effect on the ability of *in vitro* maturation of cat oocytes. Parallel to these findings, in this study the *in vitro* maturation rates (MII) in negative control and fresh groups were 55.7% and 52.4% respectively and were statistically similar (P>0.001). These findings are close to our previous IVM results (51%) and support the claim that oocytes can maintain their ability to mature *in vitro* after storage of the ovary for 24 h at 4°C. We have also determined that adding melatonin to storage medium has a negative effect on IVM rates (MII) of cat oocytes and the *in vitro* maturation rates are decreased as melatonin rates increased (P<0.001). Our results contradict the findings that the addition of melatonin in the ovarian storage medium has beneficial effects *in vitro* embryo production rate of sheep oocytes [7]. These different results may be due to the different *in vitro* conditions of the study, the different *in vitro* maturation time of cat oocytes from that of sheep, or other species-specific sensitivities.

Since mammalian gametes have high lipid concentrations, they are highly vulnerable to oxidative stress and melatonin plays an important role as an antioxidant [17]. Supplementation of superoxide dismutase (SOD) of the ovarian storage medium under cold conditions has been reported to decrease cellular apoptosis by increasing BCL-2 expression and reducing BAX expression in sheep [7]. Melatonin has been also reported to downregulate the expression of the BAX and upregulate the expression of BCL-2 by increasing intracellular glutathione and reducing ROS production in some mammals [16,24-26]. Moreover, it was reported that melatonin has a strong antioxidant effect and protect oocytes from free radicals produced during ovulation and improve oocyte maturation in many mammalian species [16]. Some researchers reported that the use of melatonin as an antioxidant in the ovary storage medium had beneficial effects on both the development of sheep oocytes and the quality of the embryos and 700 and 800 (μM) concentrations gave the best results melatonin supplementation in the long-term preservation of the sheep ovaries at even 4 or 20°C temperatures [7]. Contrary of these results, in our study the IVM rates of cat oocytes in both control and fresh group that had no melatonin, were higher than all of the melatonin added groups. Moreover, as the amount of melatonin increased, these rates also decreased (P<0.001). Interestingly, the change in expression of the BCL-2 gene was more pronounced with increasing doses of melatonin in both fresh and stored groups. However, these trends in gene expression levels were not statistically significant. This insignificance may have resulted from the timing of sampling for genetic analysis. It was observed that the similar amount of melatonin used in sheep ovaries does not provide similar benefits in cats, or even harmful to IVM abilities [17].

High amounts of melatonin in follicular fluid have been reported to support the follicle development and protect cumulus oophorus cells and oocyte [16,27,28]. It has been shown that the addition of melatonin to the *in vitro* maturation medium in rats reduces ROS, decreases the expression of pro-apoptotic BAX gene and promotes the expression of an anti-apoptotic BCL-2 gene [24]**.** Although some researchers stated that melatonin supplementation improves oocyte development *in vitro* in some mammalian species [17,25,27-29], there was no statistically significant difference in BCL-2/BAX ratio between melatonin and control groups in both fresh and stored groups (P>0.05). Both our results from our previous study [1] showing that storage of domestic cat ovaries at 4°C for up to 24 h has no adverse effect on the *in vitro* maturation ability of oocytes, as well as our findings here, may suggest that melatonin acts in a species-specific manner and that other cats. It also brings to mind the idea that it may have a different mechanism of action in cat, unlike other species. More studies with different doses and more numbers are needed to find out whether this negative effect of melatonin is due to the concentrations or to a species-specific situation.

In conclusion our present results have shown that unlike most studies in other mammals, adding melatonin (500, 750 and 1000 μM) to the ovarian storage medium had harmful effects on the viability and the structure of oocyte cell membrane in cat.

Availability of Data and Materials

All data sets collected and analyzed during the current study are available from the corresponding author (M. Evecen) on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

M. Evecen planned the present study, conducted this experiment, and wrote this manuscript; K. Ak, S. Pabuccuoğlu, S. Birler, K. Demir, S. Yağcıoğlu, R. Arıcı, A. Eser, G. Bakirer Öztürk, A. Kılıçkap, N. Ersoy and İ. Oruç, conducted and supported this experiment; E. Kömürcü Bayrak and B. Özsait Selçuk performed the genetic analyzes of the study.

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RESEARCH ARTICLE

The Effect of Different Storage Temperature on Hu Ram Sperm Parameters

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Abstract: In order to explore the most suitable temperature for the preservation of Hu ram semen at room temperature, the semen samples were placed at 15°C, 20°C, and 25°C respectively. The energy metabolism of enzyme activity and triphosphate adenosine (ATP) was detected in the process of preservation. Different parameters such as functional integrity, survival time, reactive oxygen species (ROS) content, superoxide dismutase (SOD) activity, and energy metabolism of adenosine triphosphate (ATP) were detected. The results showed that within 2 days of storage, the sperm progressive motility stored at 25°C was the highest. Within 3~5 days of storage, sperm stored at 20°C have the highest progressive motility. Within 6~9 days of storage, the sperm progressive motility stored at 15°C was significantly higher than that of the other groups (P<0.05). The effective survival time and total survival time of sperm stored at 15°C reached 5.67 d and 18.73 d, respectively, which were significantly higher than those of other groups (P<0.05). On the 1st day of storage, the sperm membrane integrity was significantly higher than that of the other groups (P<0.05). Within 3~5 days of storage, the membrane integrity and acrosome integrity of sperm stored at 15°C and 20°C were significantly higher than the sperm membrane integrity and acrosome integrity stored at 25°C (P<0.05). On the 7th day of storage, the membrane integrity of sperm stored at 15°C was significantly higher than that of other groups (P<0.05). On the 5th day of storage, the level of sperm ROS stored at 15°C was significantly lower than that of group 25°C (P<0.05), SOD activity, Catalase enzyme (CAT) activity and ATP content were significantly higher than that of the other groups (P<0.05). Therefore, compared with 20°C and 25°C, 15°C was the most suitable temperature for the preservation of Hu ram semen at room temperature.

Keywords: *Temperature, Sperm, Motility parameters, Physiological characteristics, Oxidative stress*

Farklı Saklama Sıcaklıklarının Hu Koç Sperm Parametreleri Üzerine Etkisi

Öz: Hu koç sperminin oda sıcaklığında muhafazasında en uygun sıcaklığı belirlemek için, sperm örnekleri sırasıyla 15°C, 20°C ve 25°C'de bekletildi. Muhafaza sürecinde enzim aktivitesinin enerji metabolizması ve adenosin trifosfat (ATP) ölçüldü. Fonksiyonel bütünlük, yaşama süresi, reaktif oksijen türlerinin (ROS) içeriği, süperoksit dismutaz (SOD) aktivitesi ve adenozin trifosfatın (ATP) enerji metabolizması gibi farklı parametreler ölçüldü. Bulgular, 2 günlük muhafaza süresince 25°C'de bekletilen spermlerin progresif hareketliliğinin en yüksek olduğunu gösterdi. 3~5 günlük muhafaza süresince, 20°C'de bekletilen spermler en yüksek progresif hareketliliğe sahipti. 6~9 günlük depolama süresince, 15°C'de bekletilen spermlerin progresif motilitesi diğer gruplara göre önemli ölçüde daha yüksekti (P<0.05). 15°C'de muhafaza edilen spermlerin etkin canlı kalma süresi ve toplam canlı kalma süresi, diğer gruplardan önemli ölçüde daha yüksekti ve sırasıyla 5.67 gün ve 18.73 güne ulaştı (P<0.05). Muhafazanın 1. gününde, sperm membran bütünlüğü diğer gruplara göre anlamlı derecede yüksekti (P<0.05). Muhafazadan sonraki 3~5 gün içinde, 15°C ve 20°C'de saklanan spermin membran bütünlüğü ve akrozom bütünlüğü, 25°C'de saklanan spermin membran bütünlüğü ve akrozom bütünlüğünden önemli ölçüde daha yüksekti (P<0.05). Muhafazanın 7. gününde 15°C'de saklanan spermin membran bütünlüğü diğer gruplara göre anlamlı derecede yüksekti (P<0.05). Muhafazanın 5. gününde, 15°C'de saklanan spermin ROS seviyesi, 25°C'de saklanan grubtan anlamlı derecede (P<0.05) düşük iken, SOD aktivitesi, katalaz enzim (CAT) aktivitesi ve ATP içeriği diğer gruplardan önemli ölçüde daha yüksekti (P<0.05). Dolayısıyla, 20°C ve 25°C ile karşılaştırıldığında, Hu koç sperminin korunması için en uygun oda sıcaklığı 15°C olarak saptandı.

Anahtar sözcükler: *Sıcaklık, Sperm, Motilite parametreleri, Fizyolojik özellikler, Oksidatif stres*

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Introduction

Hu sheep is a world-famous high fecundity sheep breed and a first level protected local livestock breed in China [1,2]. Hu sheep is known for their high fertility and the breeding scale is gradually expanding [3-5].

In order to improve the reproduction efficiency of Hu sheep, to make full use of the semen of excellent breeding males, to reduce breeding costs, and to avoid the spread of diseases caused by natural mating; Artificial Insemination (AI) has been applied to the breeding of Hu sheep in actual production [6,7]. Semen preservation is the core part of AI; which is mainly divided into room temperature preservation, 4°C and cryopreservation [8]. Because sheep sperm membranes contain a large amount of unsaturated fatty acids, they are susceptible to irreversible low temperature damage, destroying the complete structure of sperm, causing sperm to lose physiological functions and internal material loss, thereby affecting the quality of semen preservation [9]. On the other hand, cryopreservation not only requires expensive instruments, but also high technical requirements, which limits its application in production practice. Cryopreservation causes a certain cold shock to sperm, and there are also certain technical requirements. Therefore, in actual production, room temperature preservation is widely used, which is more conducive to improve the utilization rate of genetic resources of breeding rams [10]. Optimum temperature is essential for semen preservation. When the storage temperature of semen increases, the metabolism and respiration of sperms enhance, and the movement and energy consumption increase. When the temperature rises to a certain level, the decomposition of the sperm itself will increase the ammonia content and increase the pH of the semen, which will further enhance the metabolism and respiration of the sperm [11]. When the temperature rises, the reproduction speed of bacteria in semen will also increase, producing a large amount of metabolic waste, damaging sperm cells, and affecting the preservation quality of semen [12]. When the temperature drops to a certain level, it will affect the physiological function of the sperm or destroy the sperm structure [13]. For example, the optimal temperature for storage of pig semen at room temperature is 17°C. 15°C~25°C belong to the range of room temperature preservation, but there is no uniform standard for the optimum temperature for storage of Hu ram semen at room temperature.

In this experiment, different storage temperatures (15°C, 20°C, and 25°C) were assumed to store Hu ram semen at room temperature. Aim was to detect and analyze of Hu ram sperm motility parameters such as total motility, progressive motility, survival time and membrane integrity. Other functional integrity parameters such as ROS level, SOD activity oxidative stress parameters and ATP content under different room temperature storage of semen. The basic object of the study was to obtain optimal temperature for storage of Hu ram semen at room temperature.

Material and Methods

Ethics Statement

All animal procedures confirm to the guidelines and regulatory standards of the Animal care committee of the Yangzhou University (Approval ID: SYXK [Su] 2017-0044).

Experimental Design

Five experiments were conducted to evaluate the effects of different temperatures on semen preservation of Hu rams at room temperature.

Experiment 1: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the motility parameters of sperm. Sperm total motility, progressive motility, and kinematic parameters of the three groups were evaluated.

Experiment 2: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the survival time of sperm. Effective survival time, and total survival time of the three groups were evaluated.

Experiment 3: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the physiological characteristics of sperm. Membrane integrity, and Acrosome integrity of the three groups were evaluated.

Experiment 4: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the antioxidant parameters of sperm. ROS, Malondialdehyde (MDA), SOD, and CAT of the three groups were evaluated.

Experiment 5: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the ATP of sperm.

Preparation of Semen Extender and Semen Collection

The extender consisted of 15.35 g Tris, 10.00 g fructose, 8.20 g citric acid and 250.000 IU each of penicillin and streptomycin in 500 mL distilled water.

Semen samples were collected from three Hu rams aged two years with the aid of an artificial vagina. A total number of 50 ejaculates were collected from three rams. The collected semen volume of each ram was about 1.0 mL every time. Sperm concentration reached $2.3x10^9$/mL. The three rams were raised in the experimental sheep farm of Yangzhou University. The rams were fed 0.2 kg concentrate twice a day, and *ad libitum* hay and water. The experimental farm is equipped with licking bricks

for sheep to lick freely, so as to ensure sufficient minerals and microelements. There is also an open area in the experimental farm to ensure that the sheep have enough exercise. Semen samples were evaluated for sperm motility using computer-assisted sperm analyzer (CASA). Ejaculates showing >75% motility were pooled to minimize individual differences.

Dilution and Evaluation of Semen

The pooled fresh semen samples were split into three equal fractions in different test tubes and diluted at room temperature with a Tris extender. The processed semen was stored respectively in a 15°C, 20°C, and 25°C incubator and the semen was gently flipped every day.

Evaluation of Sperm Motility

Semen samples were evaluated for sperm kinetics using CASA (ML-608JZ II Mailang, Nanning, China).

Evaluation of Sperm Survival Time

The time when the sperm progressive motility is above 60% is called the effective survival time. The time when all sperm died is called the total survival time.

Evaluation of Sperm Physiological Characteristics

The hypo-osmotic swelling test (HOST) was used to evaluate the sperm membrane integrity. 10 μL of preserved semen and 100 μL of hypo-osmotic solution were mixed and incubated at 37°C for at least 30 min. Five μL of suspension was loaded on a slide and 200 cells with swollen and non-swollen tails were counted as sperm with membrane integrity and non-integrity respectively, under a 400x phase-contrast microscope. Acrosome integrity was detected by Coomassie brilliant blue staining. Fifty μL of preserved semen and 1 mL of 4% paraformaldehyde were mixed and fixed at room temperature for 10 min. After centrifugation at 1500x g for 5 min, the supernatant was discarded, and 10 μL of semen taken to make a smear. After air-drying, this was stained with Coomassie Brilliant Blue dye for at least 30 min, rinsed with water, and air-dried. Then 200 cells with head stained blue and unstained blue were counted as sperm with acrosome integrity and non-integrity under a 1000x oil immersion.

Evaluation of Sperm Antioxidant Parameters and ATP Content

ROS level of sperm was measured using a ROS Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instruction. The ROS level was expressed by the fluorescence intensity. Fluorescence intensity of DCF (488 nm excitation and 525 nm emission for DCF) was detected by a multifunctional microplate reader.

The MDA content in the semen was measured using a Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Absorbance at 532 nm was detected by a multifunctional microplate reader. Finally, the results were obtained according to the standard curve.

SOD activity of sperm was measured using a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Absorbance at 450 nm was detected by a multifunctional microplate reader. Finally, we got the result according to the formula in the instruction.

CAT activity in the semen was measured using a CAT Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Finally, we got the result according to the formula in the instruction.

The ATP content in the semen was measured using ATP Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Absorbance at 636 nm was detected by a multifunctional microplate reader. Finally, we got the result according to the formula in the instruction.

Statistical Analysis

Data were analyzed using SPSS 25.0 software. Significance was set at $P<0.05$ unless otherwise specified. The results were expressed as the Mean ± SEM. One-way ANOVA tests were performed to assess the difference in these parameters.

Results

Effects of Different Temperature on Sperm Motility

The effects of different temperature on Hu ram sperm motility during liquid storage were shown in *Fig. 1*. Sperm total and progressive motility decreased with the increasing storage time *in vitro*. After 6 days preservation, the total motility and progressive motility of sperm storage at 15°C were higher than those of other groups ($P<0.05$).

Effects of Different Temperature on Sperm Kinematic Parameters

The effects of different temperature on Hu ram sperm kinematic parameters during liquid storage were shown in *Table 1*. Within 6~9 days, the Straight line velocity (VSL), Curvilinear velocity (VCL), Average path velocity (VAP), Amplitude of lateral head displacement (ALH) and Average motion degree (MAD) in the 15°C group were higher ($P<0.05$) than those in other groups.

Effects of Different Temperature on Sperm Survival Time

As shown in *Fig. 2*, the 15°C group showed the highest effective survival time and total survival time among all

Table 1. *Effects of different temperature on sperm kinematic parameters of Hu ram*

Testing Index	Storage Time (days)	Temperature		
		15°C	20°C	25°C
VSL (μm/s)	0	31.23±0.28	31.61±0.42	31.35±0.35
	1	31.70±0.67	30.92±0.81	29.43±0.87
	2	30.76±0.98	28.63±1.00	28.30±0.40
	3	27.88±0.23^{b}	29.85±0.60^{a}	30.89±0.37^{a}
	4	26.41±0.39^{b}	28.58±0.41^{a}	26.52±0.62^{b}
	5	26.67±0.22^{a}	26.67±0.42^{a}	19.17±0.08^{b}
	6	25.32±0.71^{a}	21.37±0.22^{b}	2.78±2.78^{c}
	7	26.14±0.05^{a}	19.01±0.82^{b}	2.72±1.40^{c}
	8	22.73±0.65^{a}	17.28±1.05^{b}	0^{c}
	9	25.41±0.47^{a}	11.60±0.86^{b}	0^{c}
VCL (μm/s)	0	61.06±0.27	59.93±0.36	60.44±0.37
	1	58.75±0.96	59.15±1.24	57.72±1.42
	2	58.76±1.52	55.02±3.26	55.64±0.90
	3	56.71±1.51^{b}	62.19±0.89^{a}	65.63±1.23^{a}
	4	56.27±1.78	60.46±0.94	59.68±1.42
	5	56.74±0.49^{a}	58.19±0.77^{a}	44.84±0.53^{b}
	6	59.77±2.54^{a}	51.37±0.05^{b}	4.73±4.73^{c}
	7	57.52±0.55^{a}	42.32±1.65^{b}	3.55±1.81^{c}
	8	51.58±1.62^{a}	39.07±2.44^{b}	0^{c}
	9	55.90±0.01^{a}	26.10±1.61^{b}	0^{c}
VAP (μm/s)	0	44.04±1.05	45.33±0.66	46.37±1.21
	1	41.54±0.68	41.83±0.88	40.81±1.01
	2	41.55±1.07	38.9±2.30	39.35±0.64
	3	40.10±1.07^{b}	43.97±0.63^{a}	46.41±0.87^{a}
	4	39.79±1.26	42.75±0.67	42.20±1.00
	5	40.12±0.35^{a}	41.15±0.55^{a}	31.71±0.38^{b}
	6	42.26±1.79^{a}	36.32±0.04^{b}	3.34±3.34^{c}
	7	40.67±0.39^{a}	29.92±1.17^{b}	2.51±1.28^{c}
	8	36.47±1.14^{a}	27.64±1.72^{b}	0^{c}
	9	39.53±0.01^{a}	18.4±1.145^{b}	0^{c}
ALH (μm)	0	18.24±0.43	18.78±0.27	19.21±0.50
	1	17.21±0.28	17.32±0.36	16.90±0.42
	2	17.21±0.44	16.12±0.96	16.30±0.26
	3	16.61±0.44^{b}	18.22±0.26^{a}	19.22±0.36^{a}
	4	16.48±0.52	17.71±0.28	17.48±0.42
	5	16.62±0.14^{a}	17.04±0.23^{a}	13.13±0.16^{b}
	6	17.51±0.74^{a}	15.05±0.01^{b}	1.38±1.38^{c}
	7	16.85±0.16^{a}	12.39±0.48^{b}	1.04±0.53^{c}
	8	15.11±0.48^{a}	11.45±0.71^{b}	0^{c}
	9	16.38^{a}	7.65±0.47^{b}	0^{c}
MAD (°/s)	0	146.23±14.22	149.79±5.73	145.25±6.33
	1	97.56±13.87	96.64±1.69	112.7±11.17
	2	93.59±4.27	102.46±6.17	92.78±2.35
	3	74.15±1.07	79.58±2.48	80.70±3.40
	4	69.08±6.08	97.27±13.94	73.50±8.03
	5	62.47±1.68^{a}	67.63±4.92^{a}	38.8±1.656^{b}
	6	36.38±2.64^{a}	23.50±3.99^{b}	6.3±3.295^{c}
	7	31.97±2.49^{a}	21.0±0.605^{b}	3.97±3.97^{c}
	8	28.92±1.28^{a}	17.56±2.47^{b}	0^{c}
	9	19.63±1.88^{a}	11.14±3.08^{b}	0^{c}

Data are expressed as the Mean ± SEM. Letter difference (a-c) means significant difference (P<0.05)

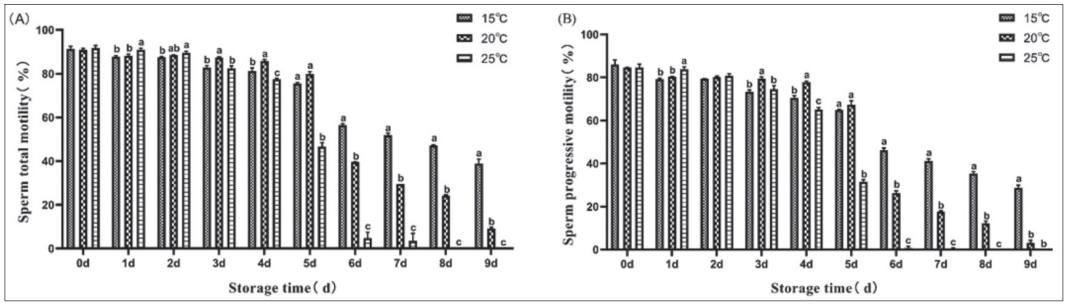

Fig 1. Effects of different temperature on sperm motility of Hu ram. (A) The percentage of sperm total motility in the 15°C, 20°C and 25°C groups; (B) The percentage of sperm progressive motility in the 15°C, 20°C and 25°C groups. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

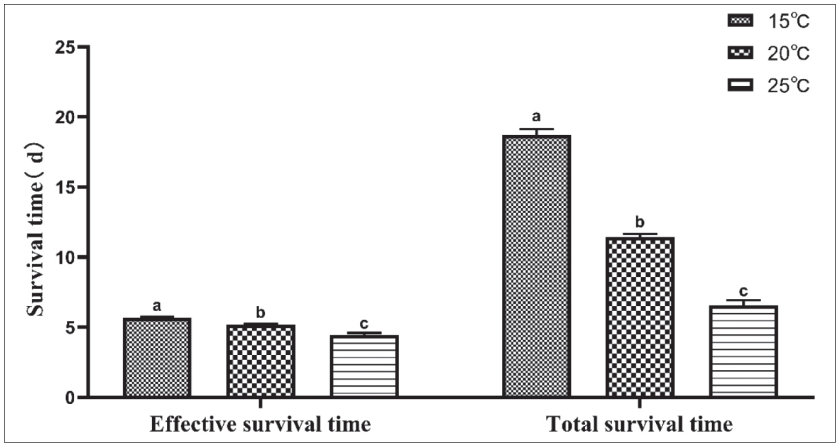

Fig 2. Effects of different temperature on sperm survival time of Hu ram. The time when the sperm progressive motility is above 60% is called the effective survival time; when the sperm total motility drops to 0, the time when all sperm die is called the total survival time. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

groups (P<0.05). The effective survival time and total survival time of sperm were 5 days and 18 days, respectively.

Effects of Different Temperature on Sperm Physiological Characteristics

Microscopic examination after HOST incubation was shown in *Fig. 3-C*. There were three types of sperm tail: C, D, and E, in which the two types of tail, C and D which are curled represented intact membrane sperm, and the tail type E that is non curled represented sperm with damaged membrane. The 15°C group had the highest membrane integrity on the 7th day (P<0.05).

Microscopic examination after Coomassie brilliant blue staining was shown in *Fig. 3-D*. There were two types of sperm head: A and B. If the sperm head was blue, then the acrosome was intact (A). If the sperm head was unstained, then the acrosome was not intact (B). As shown in *Fig. 3-B*, the 25°C group had the lowest acrosome integrity within 5~7 days (P<0.05).

Effects of Different Temperature on Sperm Antioxidant Parameters

The effects of different temperature on Hu ram sperm ROS level during liquid storage were shown in *Fig. 4-A*. The sperm ROS level of the 15°C group was lower (P<0.05) than that of 25°C group on the 5th day. As shown in *Fig. 4-B*, on the 5th day, the content of MDA in 15°C group was higher than that in other groups (P<0.05). It can be seen in *Fig. 4-C* that 15°C group had the highest SOD activity on the 5th day (P<0.05). As shown in *Fig. 4-D*, on the 5th day, CAT activity of the 15°C group was higher (P<0.05) than that of 20°C group.

Effects of Different Temperature on Sperm ATP Content

The effects of different temperature on Hu ram sperm ATP content during liquid storage on the 5th day were shown in *Fig. 5*. ATP content of the 15°C group was significantly higher (P<0.05) than that of other groups.

Discussion

This study analyzed the effects of three different storage temperatures *in vitro* on the motility parameters of sperms such as sperm total motility, survival time, functional integrity such as membrane integrity, oxidative stress parameters such as ROS level, enzyme activities such as SOD activity and ATP content. Within 2 days of storage,

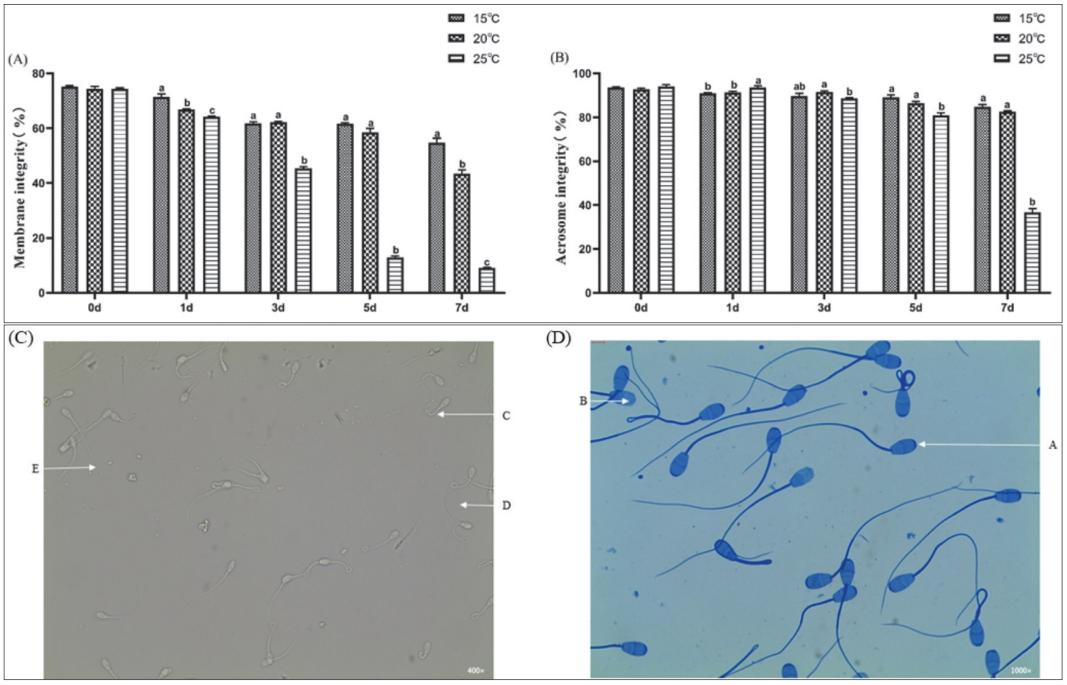

Fig 3. Effects of different temperature on sperm physiological characteristics. (A) The percentage of sperm membrane integrity in the 15°C, 20°C, and 25°C groups; (B) The percentage of sperm acrosome integrity in the 15°C, 20°C, and 25°C groups; (C) Morphology of curly tail of sperm in HOST; C, D: Sperm with intact membrane; E: Sperm with damaged membrane; (D) Acrosome morphology of sperm stained with Coomassie brilliant blue; A: Sperm with intact acrosome; B: Sperm with damaged acrosome. The bars represent the standard error. Letter difference (a-c) means significant difference ($P<0.05$)

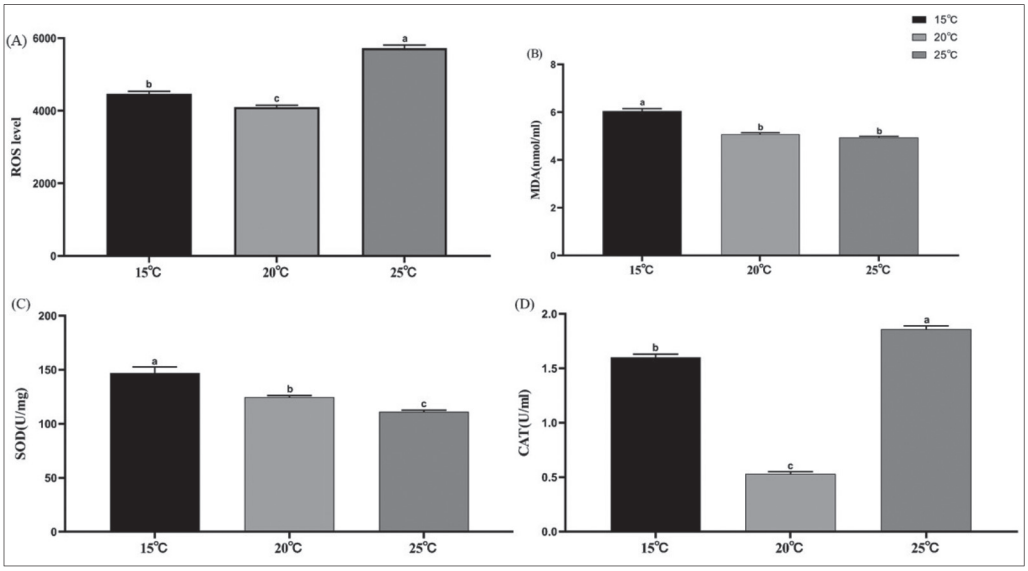

Fig 4. Effects of different temperature on sperm antioxidant parameters. (A) The ROS level of sperm in the 15°C, 20°C, and 25°C groups on the fifth day; (B) The MDA content of semen in the 15°C, 20°C, and 25°C groups on the fifth day; (C) The SOD activity of sperm in the 15°C, 20°C, and 25°C groups on the fifth day; (D) The CAT activity of semen in the 15°C, 20°C, and 25°C groups on the fifth day. The bars represent the standard error. Letter difference (a-c) means significant difference ($P<0.05$)

sperm stored at 25°C had the highest total motility and progressive motility. This may be because in the early stage of semen preservation, higher temperature stimulates sperm activity and metabolism, and lower temperature inhibits sperm movement [11]. This result was consistent with the changes in sperm motility of Hahn et al.[14] in the study of buck sperm. The motility movement speed of buck sperm at 37°C was significantly higher than that at 20°C. The study also found that higher temperatures activate sperm metabolism. For a short time, sperm motility

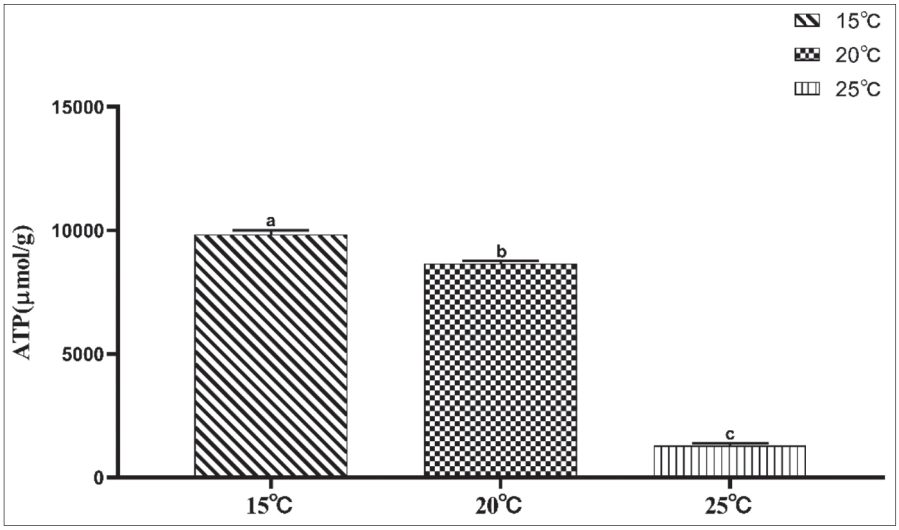

Fig 5. Effects of different temperature on sperm ATP content of Hu ram on the fifth day. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

at 37°C did not differ from sperm motility at 20°C. This differs from this study in the length of preservation. Within 3~5 days of storage, the sperm total motility and progressive motility saved at 20°C was the highest. Within 6~9 days of storage, the sperm total motility and progressive motility saved at 15°C was the highest. This may be due to the increased respiration and metabolism of sperm at higher temperatures, increased nutrients consumption and low levels of nutrients that cannot meet the metabolic activities of some sperms leading to sperm death [15]. At lower temperatures, sperm motility was restricted and nutrient consumption was slowed down, which can meet the metabolic activity of sperm. This result was consistent with the results of Verstegen et al.[16]'s research on the effect of temperature on dog sperm. High temperature will speed up sperm metabolism, increase nutrient consumption, and reduce sperm total motility. After storage for 5~6 days, the quality of semen stored under various temperature conditions decreased significantly, and the quality of semen stored at higher temperatures changed drastically. This may be due to the fact that in the early stage of semen preservation, a large amount of nutrients was consumed and the remaining nutrients cannot maintain the normal metabolism of sperm. As a result, the death rate of sperm was accelerated and the quality of semen changes dramatically [17]. Storage temperature was an important factor that affects the quality of semen, and it was vital to the survival of sperm [18].

The effective survival time and total survival time of sperm stored at 15°C were the longest. This may be because higher temperature will speed up sperm energy metabolism, faster nutrient consumption, faster bacterial reproduction in semen, and at the same time produce more metabolic waste, which has a certain toxic effect on sperm and reduces the survival time of sperm [19,20]. Within 6~9 days of storage, the VSL, VCL, and VAP of sperms stored at 15°C were the highest. Therefore, VSL, VCL, and VAP were important indicators of sperm motility parameters, which were closely related to fertilization ability [21].

Plasma membrane was the outermost structure of sperm cells and acts as a physiological barrier. The normal physiological functions, capacitation and metabolism of sperm were related to their integrity, and the integrity of the membrane can also indirectly reflect the life and death of sperm [22,23]. The membrane integrity of sperm stored at 15°C and 20°C was the highest within 3~5 days. On the 7th day of storage, the membrane integrity of sperms stored at 15°C was the highest. This result is consistent with changes in sperm total motility. In the first 5 days of preservation, the sperm acrosome integrity rate decreased slowly. On the 7th day of storage, the acrosome integrity rate of sperm stored at 25°C decreased faster. Yeste et al.[24] research shows that osmotic pressure has a great influence on the structure and physiological function of sperm. This study shows that high osmotic pressure has a great effect on the integrity of sperm membrane and acrosome. This study shows that the osmotic pressure is not only related to the solute added to the diluent, but also affected by the type and concentration of ions. In this study, it may be because that after a large number of sperm died, the dead sperm released a large number of ions into the diluent. In this experiment, the acrosome integrity rate of sperm stored at 25°C decreased rapidly. It may be that the osmotic pressure of the diluent changed with the death of a large number of sperm, resulting in a rapid decline in the acrosome integrity rate. The acrosome intact rate in this experiment was directly proportional to the sperm survival time. This result is consistent with the research results of Zhang et al.[25]'s preservation of bovine semen at room temperature. The effective survival time and sperm

motility in the optimal concentration of antioxidant group were the highest, and the sperm acrosome integrity rate was also the highest.

In the process of semen preservation, ROS produced by sperm metabolism has a certain relationship with the decline of sperm quality [26,27]. On the 5th day of storage, sperm stored at 25°C have the highest ROS level. This result was consistent with changes in sperm progressive motility. High concentrations of ROS will damage the integrity of the sperm membrane [28]. This result was consistent with the change of sperm membrane integrity. On the 5th day of storage, the semen stored at 15°C had the highest MDA. This may be due to the low mortality of sperm stored at 15°C, while the other groups have high mortality and low metabolism. This result was consistent with changes in sperm progressive motility and sperm ATP content. The level of sperm ATP content represents the level of mitochondrial activity, and it can also reflect the vitality of sperm, which is positively correlated with fertilization potential [29]. On the 5th day of storage, the sperm SOD activity was highest at 15°C. This may be due to the strong antioxidant system and high enzyme activity of the semen stored at 15°C.

Qiu [30]'s research on the liquid storage of goat semen found that 15°C has a better preservation effect on semen than 5°C and 25°C. In this experiment, compared with 20°C and 25°C, 15°C was the optimum temperature for storage of Hu ram semen at room temperature, which was consistent with the above-mentioned research results.

In conclusion, the most suitable temperature for Hu ram semen to be stored at room temperature is 15°C compared to 20°C and 25°C. The motility of sperm stored at 15°C decreased slowly throughout the preservation process. Sperm stored at 15°C had the highest effective survival time. Preserving semen at this temperature can effectively prolong the survival time of sperm and slow down the rate of sperm apoptosis.

Availability of Data and Materials

All data sets collected and analyzed during the current study are available from the corresponding author (Y. Li) on reasonable request.

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Competing Interests

None of the authors have any conflict of interest to declare.

Authors' Contributions

LZ: Conceptualization, methodology, investigation, formal analysis, writing- original draft. TS: Conceptualization, writing-original draft, writing-review & editing. YK, YW and XW: Methodology, investigation. XS: Formal analysis, writing-review & editing. YL: Writing-review & editing, visualization, supervision, project administration. All authors read, revised, and approved the final manuscript.

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Research Article

The Relationship Between Metastasis and MMP-9 in Sheep with Pulmonary Adenocarcinomas

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Abstract: This study aimed to evaluate the role of Matrix Metalloproteinase-9 in the metastasis of ovine pulmonary adenocarcinoma cases by immunohistochemical methods. Lung tissue samples were collected from 26 sheep brought to our department for routine histopathological diagnosis. Lung tissues were fixed in 10% neutral buffered formalin, following routine procedures tissues were embedded in paraffin wax. Sections from tissues were cut into 5 µm thickness and stained with Hematoxylin & Eosin to detect histopathological changes. The sections were examined under a light microscope and photographs were taken. Avidin-Biotin Peroxidase method was used as immunohistochemical staining. Tumoral cells showed acinar, papillary or mixed type growths in ovine pulmonary adenocarcinomas. Only 2 of 20 cases had metastases to lymph nodes, and tumoral cell proliferation in these metastic areas was similar to primary cancer foci. All ovine pulmonary adenocarcinomas were positive for JSRV CA and Matrix Metalloproteinase-9 immunoreactivity. Any positive expression of JSRV CA and Matrix Metalloproteinase-9 were not detected in the control group. The number of JSRV CA and Matrix Metalloproteinase-9 immune positive cells was statistically increased in the ovine pulmonary adenocarcinoma group compared to the control group. In line with the data of this study, it is thought that Matrix Metalloproteinases play a serious role in tumor metastasis in ovine pulmonary adenocarcinomas, especially Matrix Metalloproteinase-9.

Keywords: *Matrix metalloproteinase-9, Metastasis, Ovine pulmonary adenocarcinoma*

Pulmoner Adenokarsinomlu Koyunlarda Metastaz ve MMP-9 İlişkisi

Öz: Bu çalışmada, Matriks Metalloproteinaz-9'un koyunların pulmoner adenokarsinom olgularının metastazındaki rolünün immünohistokimyasal yöntemlerle değerlendirilmesi amaçlandı. Rutin histopatolojik tanı için bölümümüze getirilen 26 koyundan akciğer dokusu örnekleri alındı. Akciğer dokuları %10'luk tamponlu formalinde tespit edildi, rutin prosedürler izlenerek dokular parafin blok içine gömüldü. Dokulardan alınan kesitler 5 µm kalınlığında kesildi ve histopatolojik değişiklikleri saptamak için Hematoksilen & Eozin ile boyandı. Kesitler ışık mikroskobunda incelendi ve fotoğrafları çekildi. İmmünohistokimyasal boyama olarak Avidin-Biotin Peroksidaz yöntemi kullanıldı. Koyunların pulmoner adenokarsinomlarında tümör hücrelerinin asiner, papiller veya miks tip büyüme gösterdiği bulundu. Yirmi olgunun sadece 2'sinde lenf nodlarına metastaz vardı ve bu metastik alanlardaki tümöral hücre proliferasyonu, primer kanser odaklarına benzerdi. Tüm koyun pulmoner adenokarsinomları, JSRV CA ve Matriks Metalloproteinaz-9 immünoreaktivitesi yönünden pozitifti. Kontrol grubunda herhangi bir pozitif JSRV CA ve Matriks Metalloproteinaz-9 ekspresyonu tespit edilmedi. JSRV CA ve Matriks Metalloproteinaz-9 immün pozitif hücre sayısı, kontrol grubuna kıyasla koyun akciğer adenokarsinomu grubunda istatistiksel olarak artmıştı. Çalışmanın verileri doğrultusunda Matriks Metalloproteinazların başta Matriks Metalloproteinaz-9 olmak üzere koyun akciğer adenokarsinomlarında tümör metastazında ciddi rol oynadığı düşünülmektedir.

Anahtar sözcükler: *Matriks metalloproteinaz-9, Metastaz, Koyun pulmoner adenokarsinomu*

Introduction

Ovine pulmonary adenocarcinoma (OPA), also known as sheep pulmonary adenomatosis or jaagsiekte, is contagious lung cancer caused by *Jaagsiekte sheep retrovirus* (JSRV; family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Betaretrovirus*) [1-3]. OPA, which is seen all over the world except Australia, New Zealand and the Falkland Islands,

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causes significant economic losses (growth rate, carcass weight, milk and wool production) in the sheep industry [4,5]. Since the incubation period is quite long in naturally infected sheep, OPA has been reported generally in 2-4 year old sheep, goats and rarely in 2-month-old lambs [6,7]. Transmission of OPA occurs primarily through the aerosol route, which means that close contact with infected sheep is an important risk factor. The virus has also been detected in the milk and colostrum of infected sheep, which is a potential source of infection for newborn lambs [8]. Clinical signs in animals affected by OPA can be briefly summarized as follows; cough, dyspnea, tachypnoea, nasal discharge, loss of condition, exercise intolerance, and in some cases copious pulmonary fluid production [5,9]. JSRV causes oncogenic transformation mostly in type 2 pneumocytes and less frequently in club cells (Clara cells) and undifferentiated cells [10,11]. The World Health Organization defines OPA as a mixed adenocarcinoma with acinar, papillary, and bronchoalveolar developmental patterns, and OPA is a useful animal model for understanding viral oncogenesis in human lung adenocarcinomas due to its clinical, morphological, pathological, and histopathological similarities [7,12,13]. Metastases to regional lymph nodes occur in 0.3-25% of cases, however, distant tissue metastases are extremely rare [3,14].

Matrix metalloproteinases (MMPs) is known for their ability to impair the extracellular matrix (ECM) and basement membrane, thereby playing a vital role in promoting tumor invasion and metastasis [15,16]. MMP-9, a member of MMPs, can degrade types IV, V, VII, IX and X collagen, elastin, fibrin, fibrinogen and plasminogen, and its overexpression prognostic value for the diagnosis of distant tissue metastasis or local recurrence for human lung cancers such as invasive adenocarcinoma, non-small-cell lung cancer (NSCLC) [17-19].

The aim of the study to evaluate the role of MMP-9 in the metastasis of OPA cases by immunohistochemical methods.

Material and Methods

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK-2021/098).

Animals

Lung tissue samples were taken from 26 sheep (OPA group: 20 sheep and Control group: 6 sheep) brought to our department for routine histopathological diagnosis.

Histopathological Examinations

Lung tissues were fixed in 10% neutral buffered formalin, following routine procedures tissues were embedded in paraffin wax. Sections from tissues were cut into 5 µm thickness and stained with Hematoxylin & Eosin to detect histopathological changes. The sections were examined under a light microscope and photographs were taken.

Immunohistochemical Examinations

The routine streptavidin-biotin peroxidase complex method was used according to the manual instructions of kit (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL). Primary antibodies MMP-9 (Santa Cruz, sc-393859, Dilution Ratio: 1/100) and JSRV Capsid Protein (JSRV CA, supplied by Prof. Massimo Palmarini, Dilution Ratio: 1/1500) were used after antigen retrieval (the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven at 800 watt) and nonspecific protein blocking. The reactions were detected with 3,3'-Diaminobenzidine (DAB) chromogen. Counterstainings were conducted using hematoxylin. Then, glass slides were mounted with Entellan and a coverslip. For control sections, instead of the primary antibody, PBS was applied in drops on the sections.

Prepared slides were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyzes of the images were done with Image J Program (1.51j8). MMP-9 and JSRV CA expressions were analysed by examining three representative fields of labelled neoplastic cells with the 40X magnification. Rating systems were designated as negative (-) 0%, low (+) 1-10%, moderate (++) 11-59% or severe (+++)>60% [7].

Statistical Analysis

The significance of the MMP-9 and JSRV CA expression score difference between the OPA and control groups was evaluated with the Mann-Whitney U test. All analyzes were performed in the SPSS® (Version 18.0, Chicago, IL, USA) program. Scorings in groups were given as mean ± standard error (SE). The differences between the groups after statistical analysis were considered significant at the $P<0.05$ level.

Results

Histopathological Results

In histopathological examinations, tumoral lesions were scattered in the lung and large and small foci of proliferating neoplastic cells in the initial stage OPA cases. In advanced stages of OPA, well circumscribed tumoral growths are seen in large areas. Acinar structures formed as a result of proliferation of alveolar cubic epithelial cells and papillary extensions towards the lumen were detected. While papillary structures were more prominent in some areas, acinar structures were predominant in some areas. In addition to acinar and papillary structures, masses formed by tumoral cells in alveolar lumens were

remarkable. Cell proliferation in the bronchioles in the tumoral areas was also in patterns similar to what we observed in the alveoli. There were very few mitotic figures, anisonucleosis and anisocytosis in these cuboidal-columnar tumor cells. The interstitium of proliferating alveoli and bronchioles was enlarged due to increase in connective tissue and mononuclear cell infiltrations. The presence of large numbers of alveolar macrophages in alveolar lumens around tumoral growths were determined. Bronchopneumonia and large areas of necrosis were other important histopathological findings. In lymph nodes (2 cases in total), quite large tumoral areas showing papillary, acinar or mixed growths similar to primary foci were observed *(Fig. 1-A-D)*.

Immunohistochemical Results

All OPA cases were positive for JSRV CA and MMP-9 immunoreactivity. Any positive expression of JSRV CA and MMP-9 in the control group were not detected *(Table 1)*. The number of JSRV CA and MMP-9 immune positive cells was statistically increased in the OPA group compared to the control group *(Table 2)*. JSRV CA positive reactions were mostly in the apical cytoplasm of tumoral cells and in granular form *(Fig. 2)*. Dark brown-black MMP-9 positive reactions were in both the cytoplasm and nucleus of tumoral cells exhibiting papillary and acinar growths. In two cases that metastasized to the lymph nodes, the reactions were quite severe and concentrated in the nucleus. Parallel to the primary lesions in metastatic lymph nodes, MMP-9 expressions were intranuclear. MMP-9 immune positivity in advanced OPA cases was similar to metastatic cases. In the initial stages of OPA, the intensity of MMP-9 expressions was rather weak *(Fig. 3, Fig. 4)*.

Discussion

Anamnesis (weight loss, nasal discharge, dyspnea), clinical findings (wheelbarrow test), macroscopic and microscopic findings, immunohistochemical staining, electron microscopy, molecular methods (PCR), ultrasonography and computed tomography are very useful in the diagnosis of OPA [4,6,11,13]. The definitive diagnosis of the disease is made as a result of necropsy and histopathological examinations [4,13,20]. In histopathological examinations, papillary growths extending towards the alveolar and bronchiolar lumens in well-circumscribed tumoral areas were observed, consistent with the literature data [3,14]. In addition to papillary growths, tumoral cells form acinar structures in alveolar lumens were found as previously reported [6-8]. In this study, myxoid foci and cystic growths were also present in some cases, consistent with the literature data [3,12,14]. Similar to previous works, tumoral cells were cuboidal or columnar and mitotic figures were quite low [4,12]. As reported in previous studies, there were quite a number of alveolar macrophages in the alveolar lumens around the tumoral areas [3,13]. In addition, secondary bacterial pneumonia and large areas of necrosis were other important histopathological findings. These findings are similar to as reported by Kıran et al.[6]. In lymph nodes, quite large tumoral proliferations similar

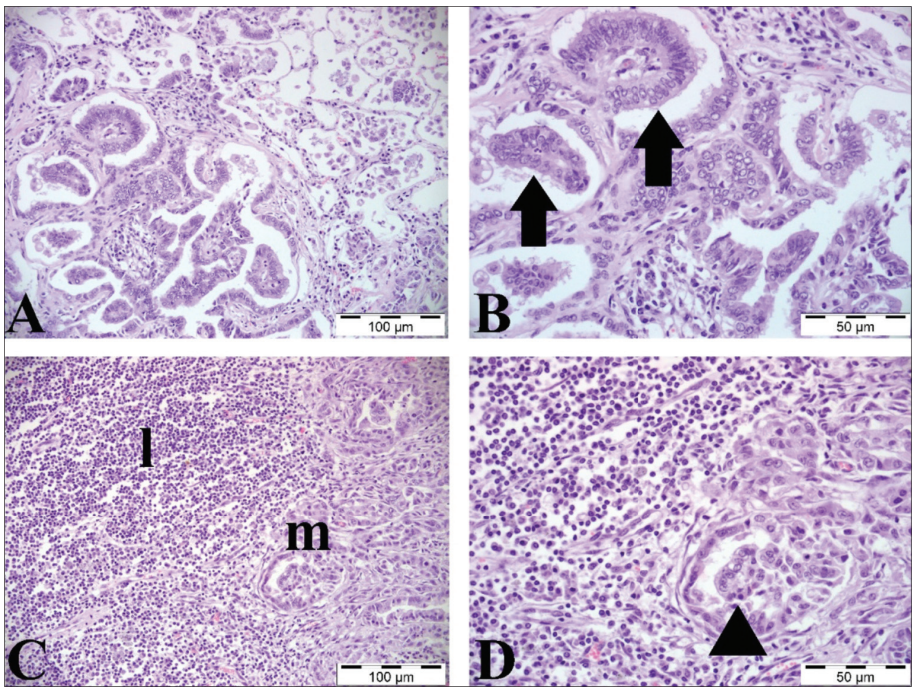

Fig 1. Lung and lymph tissue, H&E, **A**- Well circumscribed tumoral focus, **B**- Higher magnification, neoplastic cell proliferation with acinar structures in alveolar lumens *(arrows)*, **C**- Metastatic focus (m) and lymph node (l), **D**- Higher magnification, Mass consisting of tumoral cells in the metastatic focus *(arrowhead)*

Table 1. *JSRV CA and MMP-9 immunoreactivity scores of all groups*

Case No	JSRV CA	MMP-9	Metastasis
OPA Case 1	+++	+	-
OPA Case 2	+++	++	+
OPA Case 3	+++	++	+
OPA Case 4	++	+	-
OPA Case 5	++	+	-
OPA Case 6	++	+	-
OPA Case 7	++	+	-
OPA Case 8	+++	+	-
OPA Case 9	+++	+	-
OPA Case 10	+++	+	-
OPA Case 11	+++	+	-
OPA Case 12	++	+	-
OPA Case 13	++	+	-
OPA Case 14	++	+	-
OPA Case 15	++	++	-
OPA Case 16	+++	++	-
OPA Case 17	+++	+	-
OPA Case 18	+++	+	-
OPA Case 19	+++	+	-
OPA Case 20	+++	+	-
Control Case 21	-	-	-
Control Case 22	-	-	-
Control Case 23	-	-	-
Control Case 24	-	-	-
Control Case 25	-	-	-
Control Case 26	-	-	-

Table 2. *Mean ± SE values of all groups*

Groups	JSRV CA	MMP-9
Control	0±0 [a]	0±0 [a]
OPA	2.60±0.11 [b]	1.20±0.09 [b]
P value	<0.001	<0.001

[a,b] Values within a row with different superscripts differ significantly at P<0.05

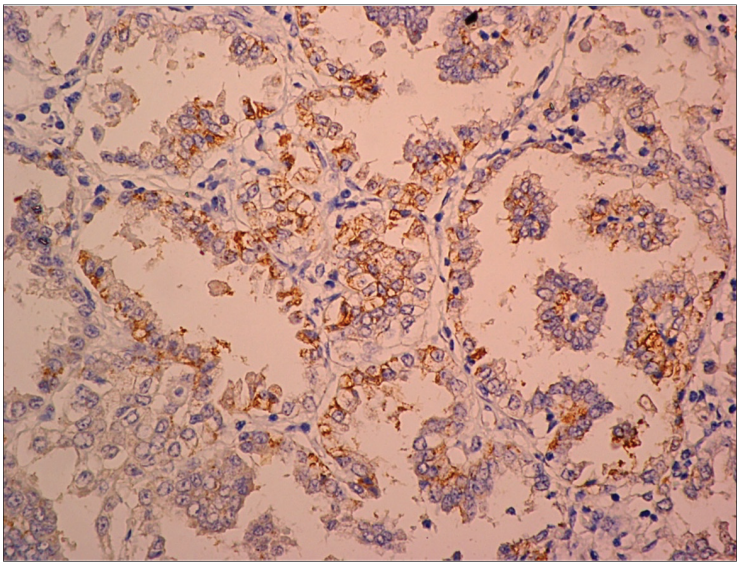

Fig 2. JSRV CA immune positive reaction in the apical cytoplasm of neoplastic cells, IHC, 20X Objective

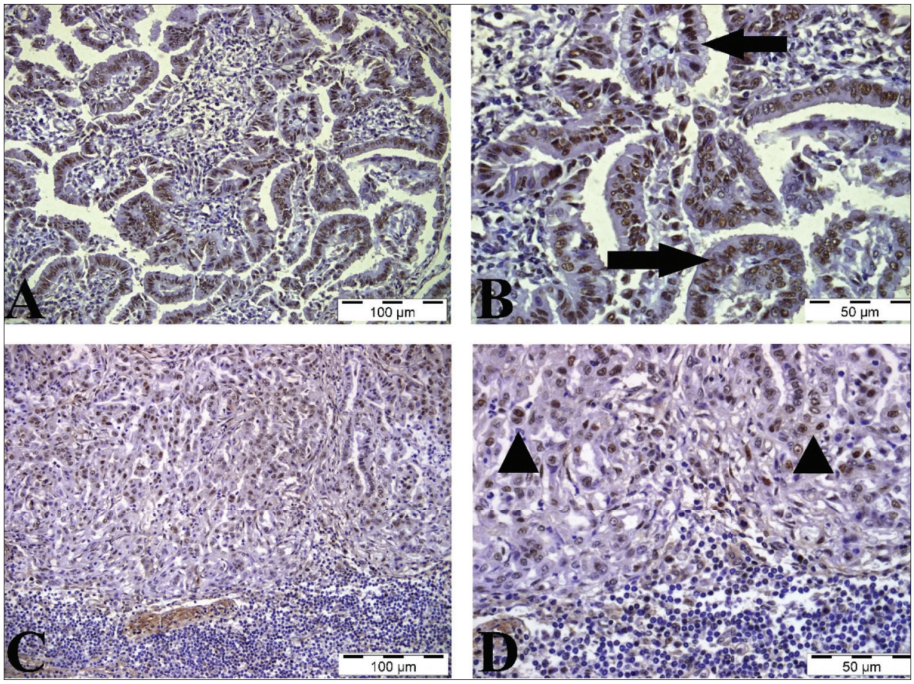

Fig 3. Lung and lymph tissue, MMP-9, IHC, **A**- Lung, tumoral area, **B**- Higher magnification, immune positive reactions *(arrows)* in the nuclei of neoplastic cells forming acinar structures in their alveolar lumens, **C**- Metastatic focus in the lymph node, **D**- Higher magnification, intranuclear MMP-9 expressions *(arrowheads)* in tumoral cells

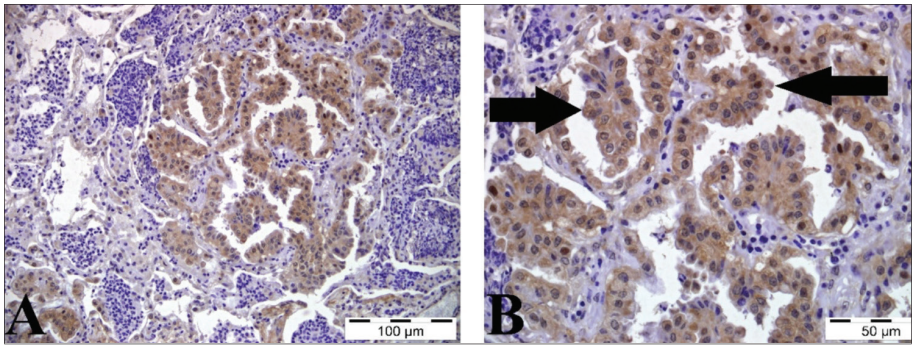

Fig 4. Lung tissue, MMP-9, IHC, **A**- Lung tissue, tumoral focus, **B**- Higher magnification, Intra-cytoplasmic yellow-brownish positive reactions in tumoral cells forming papillary proliferation and masses *(arrows)*

to primary lung foci were detected. These findings were consistent with previous studies [3,6,7,14]. All of the cases were diagnosed as OPA histopathologically were immune positive for JSRV CA expression and these immune positive reactions were in the cytoplasm of neoplastic epithelial cells as reported by different researchers [3,7,12,14].

OPA mostly metastasizes to bronchiolar and mediastinal lymph nodes. Metastasis to distant tissues such as liver, kidney, heart and skeletal muscle is very rare [1,14]. In our study, in parallel with the literature data, only 2 of 20 (10%) OPA cases had metastasis in the mediastinal lymph node [1,14,20]. Metastasis is a complex multistep process involving cell adhesion and proteolytic degradation of the ECM [16,21]. The proteolytic degradation of the ECM plays a key role in the invasion and metastasis of tumor cells [16,18,22]. MMPs, a family of zinc-dependent endopeptidases, are involved in breakdown of ECM and basement membrane, and perform critical tasks in facilitating tumoral invasion and metastasis [15,16,23]. Various researchers have associated increased MMP-9 expression in many malignancies such as NSCLC, lung adenocarcinoma, colon cancer, with cancer progression, invasion, metastasis, pathological grading and staging, and poor prognosis [15,16,24]. The increase in MMP-9 expression is a very valuable marker in the evaluation of recurrence and distant tissue metastasis in NSCLC patients, and it has been demonstrated by cell culture and clinical evaluations that tumoral invasion and metastasis decreased significantly by inhibiting MMP-9 [17,21]. Although there are a large number of studies evaluating the relationship between MMP-9 and metastasis in lung cancers in human medicine in the literature reviews, there are almost no studies evaluating the relationship between OPA and MMP-9 in veterinary medicine [20]. Mishra et al.[20] evaluated MMP-2 expressions in OPA cases by immunohistochemical methods and observed MMP-2 positive reactions in the cytoplasm of tumoral cells and in inflammatory cells. Mishra et al.[20] reported that there was no direct relationship between metastasis of OPA cases and MMP-2 expressions. In another study, Gomes et al.[22] measured MMP-2 and MMP-9 genes in lungs and cultured AECIIs of JSRV-induced pulmonary adenocarcinomas and reported no difference in MMP-2 mRNA levels in cancers compared to normal lungs. They also noted that only four cancers expressed MMP-9, while normal samples did not express this gene. Chitra et al.[23] measured MMP-9 expressions in JSRV *Env*-mediated lung adenocarcinoma invasive cell line (A549) with gelatin zymogram and noted that MMP-9 expressions were quite high. In current study, MMP-9 expression increased statistically in OPA cases compared to normal lung tissues were determined, similar to the literature data [20,22,23]. MMP-9 expressions were severe especially in metastatic OPA cases and neoplastic cells were positive for MMP-9 immunoreactivity in tumor areas in lymph nodes, similar to primary lung tumor areas. Nuclear MMP-9 staining was also detected in our study. This is a rare finding because MMP-9 expression is predominantly cytoplasmic or extracellular. Nuclear MMPs have functions such as leading to apoptosis, tissue remodeling upon injury, and cancer progression [25]. It has been reported that intranuclear MMP-9 activity degrades nuclear DNA repair proteins and causes accumulation of oxidative DNA damage in various types of cells such as neurons [26,27]. The nuclear localization of gelatinases and their nuclear substrates supports a new role for intranuclear gelatinase activity in an intrinsic apoptotic pathway [26,28], and Hill et al.[27] confirmed the nuclear localization of gelatinases and their substrates in an acute stroke injury model further supporting a novel role for intranuclear gelatinase activity in an intrinsic apoptotic pathway in neurons during acute stroke injury. In line with the data of this study, it is thought that MMPs play a serious role in tumor metastasis and cancer progression in OPA cases, especially MMP-9. In addition, intranuclear MMP-9 expressions may be related to the triggering of the intrinsic apoptosis pathway caused by JSRV and the resulting DNA damage.

In conclusion, in order to fully understand the metastatic potential of OPA cases, it is essential to evaluate all members of the MMP family in a systemic and controlled manner and to evaluate different methods such as gelatin zymogram, PCR and western blot apart from immunohistochemical methods.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

Funding Support

None.

Competing Interests

Authors declare there are no conflicts of interest in the present study.

Author Contributions

EK: Idea, concept and writing the article; HN, AY and EK: Histopathological and immunohistochemical stainings; EB, SD and EK: Histopathological and immunohistochemical analysis.

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Research Article

Evaluation of Surgical Treatment Using PRF Membrane in Deep Corneal Ulcers Accompanied by a Descemetocele in Cats: Retrospective Study (2019-2021)

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Abstract: In the present study, the results of the new generation platelet-rich fibrin (PRF) membrane graft application in deep corneal ulcers accompanying descemetocele in cats in ocular surface reconstruction surgery were evaluated, and a retrospective non-comparative study covering the period between May 2019 and August 2021 was conducted. Patients underwent routine surgical procedures for corneal ulcers, and then a new-generation autologous PRF membrane graft was transplanted to the ulcer site. Nineteen eyes were analyzed (14 males, five females). Corneal integrity and vision were achieved in all cases after treatment. No signs of infection and inflammation were noted after membrane transplantation. The PRF membrane was transplanted to the defected area with sutures generating highly effective results in eliminating the defect's urgency, supporting the cornea, and accelerating the corneal healing. Corneal integration of the PRF membrane was successful in all cases without postoperative separation or stretching. In conclusion, the PRF membrane grafting was safely and effectively utilized in the surgical treatment of deep corneal ulcers in cats with a success rate of 89.4% in descemetocele cases.

Keywords: *Cat, Corneal ulcer, Descemetocele, Graft, PRF membrane*

Kedilerde Desmatoselin Eşlik Ettiği Derin Kornea Ülserlerinde PRF Membran Kullanılarak Cerrahi Tedavinin Değerlendirilmesi: Retrospektif Çalışma (2019- 2021)

Öz: Sunulan bu çalışmada kedilerde desmatoselin eşlik ettiği derin kornea ülserlerinde yeni nesil trombositten zengin fibrin (PRF) membran grefti uygulamasının oküler yüzey rekonstrüksiyonu cerrahisindeki sonuçları değerlendirildi ve Mayıs 2019 ile Ağustos 2021 arasını kapsayan retrospektif karşılaştırmalı olmayan bir çalışma yapıldı. Kornea ülseri olan hastalara rutin cerrahi prosedürlerin ardından ülser bölgesine yeni nesil otolog PRF membran grefti nakledildi. Çalışmada on dokuz göz değerlendirildi (14 erkek, beş dişi). Tedavi sonrası tüm olgularda korneanın bütünlüğü ve gözde görme sağlandı. 10. günden itibaren tüm kedilerde göz içi basıncı ölçümü yapıldı ve tonometre oküler tonus seviyelerini rahatlıkla belirledi. Membran transplantasyonundan sonra herhangi bir enfeksiyon veya yangı belirtisi tespit edilmedi. PRF membran dikişlerle kornea defekt bölgesine nakledildi ve kusurun aciliyetini ortadan kaldırma, korneayı destekleme ve korneal iyileşmeyi hızlandırmada etkili olduğu belirlendi. PRF membranının kornea entegrasyonu, postoperatif ayrılma veya gerilme olmaksızın tüm vakalarda başarılı olduğu görüldü. PRF membran greftinin uygulandığı bu serideki desmatoselli olgularda elde edilen %89.4 başarı oranı ile kedilerde derin kornea ülserlerinin cerrahi tedavisinin yönetiminde diğer kornea rekonstrüksiyon yöntemlerine benzer şekilde güvenli ve etkili olarak kullanıldığı bulundu.

Anahtar sözcükler: *Kedi, Kornea ülseri, Desmatosel, Greft, PRF membran*

Introduction

Deep corneal ulcers and descemetocele are common disorders seen in companion animals [1,2]. In feline practice, these conditions can result from trauma, bacterial or viral (feline herpes virus) agents, tear film deficiency, decreased corneal sensitivity, inflammation, or foreign bodies [3,4]. A descemetocele refers to the anterior bulging of the Descemet membrane through a defect of overlying corneal stromal and epithelial layers [5]. The Descemet membrane

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restrains the perforation of the eye. Thus, cases with a suspected descemetocele should be handled with utmost care to avoid potential ocular perforation. Therefore, a descemetocele is considered a surgical emergency [6]. There are various reconstructive surgical procedures, such as conjunctival grafts and flaps [3,7-9], kerato-plasty [6], and the use of biomaterials [2,10,11]. However, these techniques also present with a number of disadvantages [10,11].

Platelet-rich fibrin (PRF), a second-generation platelet concentrate, was developed for use in oral and maxillo-facial surgery [12-14]. It is an autologous fibrin membrane, containing platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF-α), as thrombospondin-1, fibronectin, and vitronectin in its supporting three-dimensional fibrin structure. These growth factors and matrix proteins stimulate angiogenesis and tissue healing [15]. Besides, PRF provides mechanical support with the three-dimensional fibrin structure as a scaffold for the migrating cells [16].

The aim of this study was to evaluate the healing process of autologous PRF membrane on deep corneal ulcers associated with descemetoceles in cats.

Material and Methods

Ethical Statement

This study carried out in cats was approved by the Animal Experiments Ethics Commission of Istanbul University-Cerrahpaşa, with the decision numbered 2021/35.

Animals

The study included 19 eyes of 19 cats with different etiologies, treated with PRF membrane graft biomaterial in Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Surgery between May 2019 and August 2021. In the data analysis of each case, age, sex, affected eye, simultaneous ocular diseases, localization, diameter and vascularization of the descemetocele, vision evaluation, corneal opacity, and graft were evaluated *(Table 1)*.

Ophthalmologic Examination

All cats in this study underwent an ophthalmic examination, including a neuroophthalmological assessment, fluorescein test, Schirmer's tear test, intraocular pressure measurement, and fundus evaluation.

Preparation of PRF Membrane

In the preparation of the PRF membrane in cats, the procedures described for the preparation of the human PRF were modified and followed Can et al.[5]. Fifteen to 20 min before the surgery, 3 to 5 mL fresh blood was taken from the jugular vein of the sedated cats into two 10 mL anticoagulant-free tubes using butterfly needles (19-21 G) as quickly as possible. The tubes were centrifuged immediately at 2.700 rpm for 12 min using a Hettich 200 EBA model centrifuge. After centrifugation, three regions formed in the tube: platelet-weak plasma (PPP) at the top, platelet-rich fibrin in the middle, and red blood cells accumulated at the bottom. The resulting product was gently removed from the tube with the aid of forceps, and the red blood cells were removed using sterile scissors. The PRF clot was placed on a sterile gauze and left to release its serum (PRF-clot exudate). Each fibrin clot was gently compressed between sterile gauze pads by forced exudate extraction method to expel maximum fluid to form a membrane to be sutured to the corneal ulcer bed *(Fig. 1-A)*, and the membrane was left within the gauze to sustain releasing of the serum until it was transferred to the corneal region. Two tubes of blood were collected from each patient, and two membranes of different sizes and thicknesses were prepared.

Preparation of Surgery

Premedication was performed with 0.5-1.0 mg/kg xylazine HCl, intravenous (IV) (Basilazin, Bavet, Turkey), analgesia with 0.1-0.2 mg/kg meloxicam subcutaneous (SC) (Melox, Nobel Limited, Turkey), and antibiosis with 25-30 mg/kg ceftriaxone (IV) (Novosef, Sanofi, Turkey). General anesthesia was induced with 5 mg/kg ketamine (IV) (Alfamine, Atafen, Turkey) and maintained with isoflurane 2.0-2.5% (Forane, Abbott, Italy) and 100% oxygen.

Surgery

All necrotic and collagenolytic tissues at the edge of the corneal ulcer where the PRF membrane would be placed were debrided using a blunt corneal scalpel, and the visualization of the healthy cornea at the edges of the ulcer was achieved for suture placement. One of the grafts was prepared to be 1-2 mm larger than the corneal defect, and the second was larger to cover the other graft. The first small and thick graft was placed in the corneal ulcer bed and sutured to the margin of the healthy cornea by four to five cardinal simple interrupted sutures with 8/0 polyglactin 910 (Vicryl, Ethicon); then, the thinner and larger second graft was sutured to the healthy cornea, completely covering the first graft filling the defect *(Fig. 1-B)*. Utmost care was taken to avoid the formation of corneal perforation when sewing the graft to the healthy cornea on the descemetocele edge of the membrane, especially for the first layer. A nictitating membrane flap (NMF) was placed using 2/0 polyglactin 910 (10 days effective) (Vicryl, Ethicon) to prevent the de-hydration of the PRF membrane and damage from eyelid movements and to provide mechanical protection against external factors.

Postoperative Management

Systemic meloxicam (Metacam, Boehrienger Ingelheim,

Table 1. *The patient's signalment, etiology of descemetocele, summary of surgical and visual outcomes of the cats*

No	Breed	Age	Sex	Eye	Lesion Localization	Size of Lesion (mm)	Vascularization	Associated Findings	Follow-up Period	Clinical Outcome	Comments
1	DSH	3 M	M	R	Paracentral	OD: 6-8	+++	Corneal vascularization and diffuse corneal edema	2 months	Visual	Mild, focal corneal fibrosis+ anterior synechia
2	DSH	4 M	M	L	Paracentral	OS: 8-11	++	Corneal vascularization and local edema	3 months	Visual	Severe focal corneal fibrosis and anterior synechia
3	DSH	3 M	M	R	Central	OS: 3-4	+	Diffuse corneal edema, vascularization	3 months	Visual	Moderate, focal corneal fibrosis and anterior synechia
4	DSH	4 M	M	R	Paracentral	OS: 5-6	++	Diffuse corneal edema and vascularization+ upper eyelid entropion	1.5 months	Visual	Mild corneal fibrosis
5	DSH	3 Y	M	R	Paracentral	OD: 6-7	++	Corneal edema and vascularization	3 months	Visual	Mild corneal fibrosis
6	DSH	3 M	M	R	Central	OD: 3-4	+	Perilimbal corneal vascularization, diffuse edema	2 months	Visual	Transparent
7	DSH	6 M	M	L	Paracentral	OS:2-4	++	Corneal edema, vascularization, granulation tissue	8 months	Visual	Transparent
8	DSH	3 M	M	L	Central	OS: 2-3	+	Perilimbal and corneal vascularization, diffuse edema	2 months	Visual	Mild corneal fibrosis
9	DLH	8 Y	M	R	Central	OD: 9-11	+++	Diffuse corneal edema, granulation tissue	2 months	Visual	Mild corneal fibrosis
10	DLH	5 Y	F	L	Central	OS:2-3	+	PPM, corneal edema, mild corneal vascularization	6 months	Visual	Moderate corneal fibrosis
11	DSH	2 Y	M	L	Central	OD: 3-5	++	Corneal edema and vascularization	1.5 months	Visual	Mild corneal fibrosis
12	DSH	3 M	M	R	Central	OD: 4-6	+	Diffuse corneal edema, and vascularization	1 month	Visual	Mild corneal fibrosis
13	DLH	6 M	M	R	Central	OD: 3-4	+	Focal corneal edema, vascularization	2 months	---	Mild corneal fibrosis
14	DSH	1.5 Y	F	R	Central	OD: 3-4	+	Focal corneal edema and vascularization	5 months	Visual	Transparent
15	DSH	6 M	M	L	Central	OS: 2-4	+++	Corneal edema and vascularization	2 months	Visual	Transparent
16	DSH	2 M	F	L	Central	OS: 3-4	+	Diffuse corneal edema and vascularization	2 months	Visual	Transparent
17	DLH	4 M	M	R	Central	OD: 4-5	+	Mild corneal vascularization and diffuse edema	1 month	Visual	Transparent
18	DSH	5 Y	F	L	Central	OS: 7-10	++	Adverse corneal vascularization, focal corneal edema	3 months	Visual	Adverse corneal fibrosis
19	DSH	3 M	F	L	Central	OS: 6-8	++	Mild corneal vascularization and edema	1 month	Visual	Mild corneal fibrosis

DSH: *Domestic Shorthair,* ***DLH:*** *Domestic Longhair,* ***M:*** *Male,* ***F:*** *Female,* ***R:*** *Right,* ***L:*** *Left,* ***M:*** *Month,* ***Y:*** *Year*

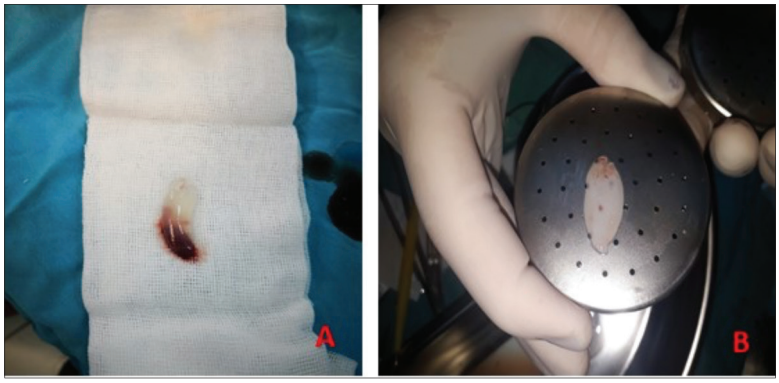

Fig 1. A- Clinical view of PRF clot and red blood cells accumulated at the bottom, **B-** The appearance of the PRF membrane after pressing between gauze

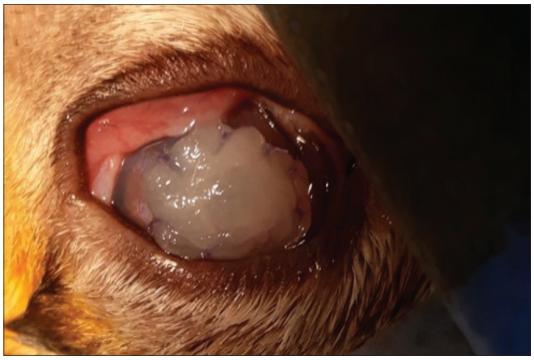

Fig 2. The PRF membrane graft immediately after surgery of the first case. The immobilization of the PRF membrane over the defective zone of the cornea with 8-absorbable suture

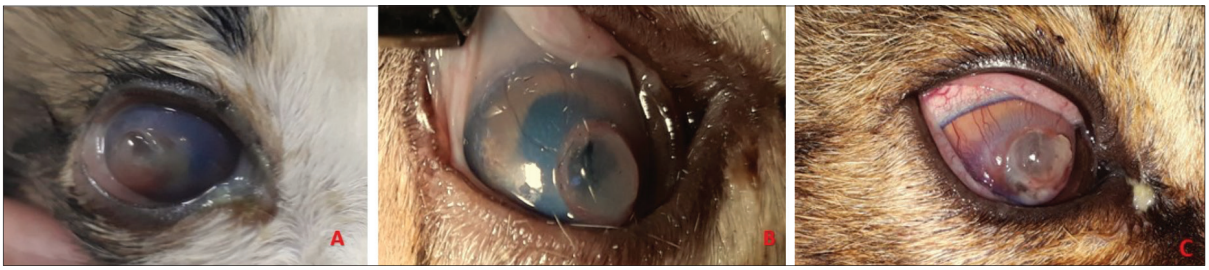

Fig 3. A- The initial ophthalmic examination (a) in case 1, **B-** Central desmatocele in the third case of the right eye (a), **C-** Photo 5 of the case before the operation, desmatocele and surrounding corneal vascularization

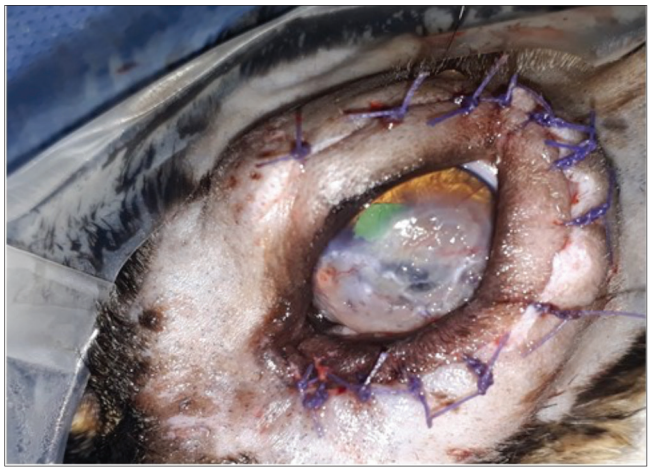

Fig 4. The left eye of case 2, 10 days post surgery, with corneal neovascularizaton and the remodeling of stroma. Correction of entropion with the modified Hotz-Celsus technique

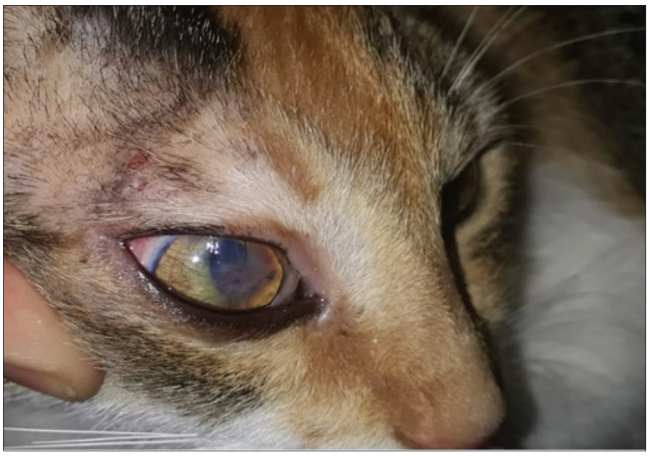

Fig. 5. The surgical results 2 weeks postoperatively in case 14, moderate corneal neovascularization and fibrosis

Germany) at 0.1-0.2 mg/kg/day PO and ceftriaxone (Novosef, Sanofi, Turkey) at 20-25 mg/kg IM once daily was administered for five days. Topical ofloxacin (Exocin, Abdi Ibrahim, Turkey) and sodium hyaluronate (Eyestil, Sifi, Turkey) were applied hourly as one to two drops for the first three days, followed by five times a day for ten days. After the NMF sutures were removed, the drops were applied four times a day for two weeks. Topical artificial tear lubricant carbomer (Thilo tears, Alcon, Turkey) was applied three times a day for about three weeks to moisten the ocular surface. Topical dexamethasone (Tobradex, Alcon, Turkey) was applied three times a day for 10 days, followed by two times for one week, and once a week; once the fibrin membrane dissolved, granulation tissue formed, and epithelialization was initiated. The topical lubricant gel was continued to be used twice a day for at least one more month.

Follow-up

NMF sutures were removed after 10 days in all cats. Ten and fourteen days after the operation, the changes in the

ocular surface, fibrin membrane, corneal vascularization, and granulation tissue were evaluated. Corneal transparency was scored on a scale of 1 to 4 (1: clear; 2: hazy; 3: moderate opacity; 4: severe opacity hampering inspection of the inner ocular structures from that site). The patients were followed up for about 8 months in terms of any recurrence, corneal perforation, increased opacity, loss of vision, and pigmentation in the ulcer region.

Results

Animals

All the affected cats were of domestic shorthair breed: fourteen intact males (one castrated), two spayed, and three intact females. The mean age at treatment was 1.1 year (ranging from 3 months to 5 years).

The Findings of the Preoperative Ophthalmological Examination

The lesion was unilateral in all cats with deep corneal ulcers accompanied by a descemetocele (n = 19) *(Fig. 2; Fig. 3-A,B,C; Fig. 4; Fig. 5)*, affecting the right cornea in 10 cats, and the left cornea in nine. The width and time course of the corneal ulcer differed in each animal; therefore, the graft sizes varied from 2 to 11 mm. Bullous keratopathy was present in 6 of 19 eyes, accompanied by corneal vascularization, edema and keratomalacia (no: 1,4,5,6,15,20). There was simultaneous keratomalacia in 4 eyes (no: 7,11,12,17), and diffuse hyphema was also noted in the anterior chamber in a single case. Based on the visual evaluation results, only 7 eyes (no: 2,3,7,10,11,12,16) (7/19 eyes, 36.84%) showed a clear presence of visual function before surgery. The menace response was negative in 10 eyes (no: 1,6,8,9,13,14,15,17,18,19), (10/19 eyes, 52.63%), and the visual function was considered uncertain in a single case (1/23 eyes; 5.26%). The dazzle reflex and pupillary light reflex were indistinct in twelve affected eyes while present in the other healthy eye. Indirect ophthalmoscopic examination of the affected eyes was achieved merely three cases (no: 3, 7,16), revealing no fundus abnormalities *(Table 2)*.

Short-Term Follow-Up: Up to 30 Days Postoperative Clinical Findings

All surgeries were performed without complications. The patients' pain, ocular discharge, and clinical complaints were determined to have decreased a few days after the operation. At the initial control visit 10 days after surgery, NMF was removed in all animal patients and no complications were observed. Vascular response to the corneal injury, edema, and epithelial proliferation, indicating an integrated cornea-PRF membrane, were prominent in all cases *(Fig. 6)*. Marked superficial and deep peripheral corneal neovascularization were noted, extending to the graft edges. Corneal vascularization was

Table 2. *Preoperative ophthalmological examination findings*

Case No	Menace Response	Dazzle Reflex	Pupillary Light Reflex	Palpebral Reflex	Intraocular Pressure	Fluorescein Dye	Schirmer Tear Test	Fundus Evaluation
1	A	UN	UN	N	10	+	17	A
2	N	N	N	N	15	+	14	A
3	N	N	N	N	14	-	20	N
4	UN	UN	UN	N	12	+	15	A
5	A	UN	UN	N	16	+	16	A
6	A	UN	UN	N	A	+	12	A
7	N	N	N	N	17	+	22	N
8	A	UN	UN	N	12	+	18	A
9	A	UN	UN	N	16	-	16	A
10	N	N	N	N	12	+	18	A
11	N	N	N	N	18	+	24	A
12	N	N	N	N	16	+	22	A
13	A	UN	UN	N	A	+	14	A
14	A	UN	UN	N	8	-	15	A
15	A	UN	UN	N	7	-	18	A
16	N	N	N	N	10	-	16	N
17	A	UN	UN	N	8	+	18	A
18	A	UN	UN	N	12	-	20	A
19	A	UN	UN	N	18	-	15	A

A: *Absent,* ***N:*** *Normal,* ***P:*** *Partial.* ***UN:*** *Uncertain*

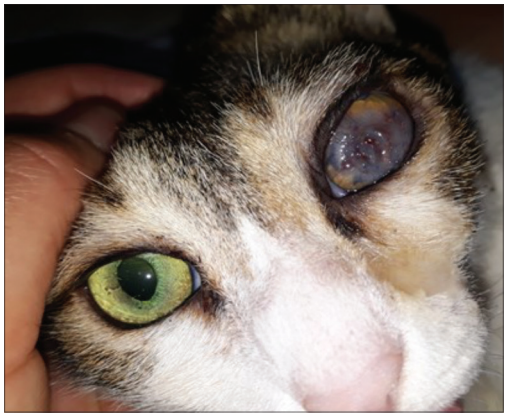

Fig 6. Anterior view of the left eye at 2 weeks after surgery

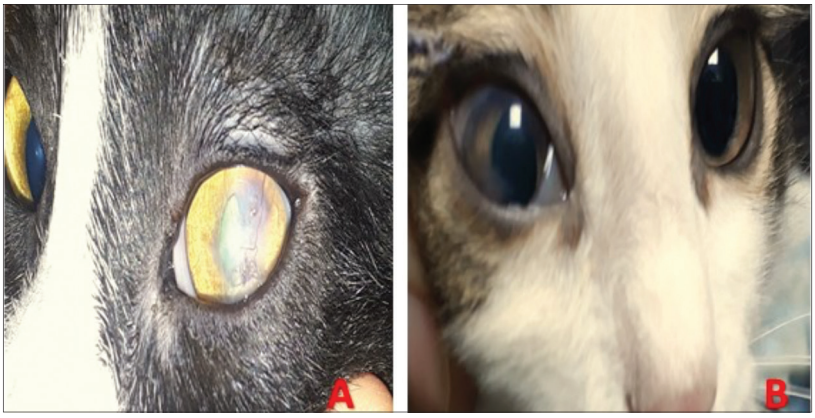

Fig 7. A- Appearance of the eye 3 weeks after surgery in case 15, **B**- Appearance 3 weeks after surgery in case 1

Table 3. *Postoperative ophthalmological examination findings*

Case No	Postoperative (2 weeks)								Postoperative (4 weeks)							
	MR	DR	PLR	PR	IP	FD	STT	FE	MR	DR	PLR	PR	IP	FD	STT	FE
1	N	N	N	N	11	+	14	A	N	N	N	N	13	-	16	N
2	N	N	N	N	14	+	15	A	N	N	N	N	16	-	18	P
3	N	N	N	N	12	-	17	N	N	N	N	N	17	-	18	N
4	N	N	N	N	11	-	15	A	N	N	N	N	18	-	16	N
5	UN	UN	UN	N	13	+	17	A	N	N	N	N	17	-	20	N
6	N	N	N	N	15	-	15	A	N	N	N	N	15	-	15	N
7	N	N	N	N	12	+	18	A	N	N	N	N	14	-	20	N
8	N	N	N	N	16	-	17	N	N	N	N	N	16	-	18	N
9	A	UN	UN	N	13	+	15	A	N	N	N	N	15	-	16	N
10	N	N	N	N	15	-	18	A	N	N	N	N	17	-	20	N
11	N	N	N	N	12	+	20	A	N	N	N	N	12	-	18	N
12	N	N	N	N	10	-	27	A	N	N	N	N	14	-	27	N
13	N	N	N	N	11	-	20	A	N	N	N	N	12	-	16	N
14	N	N	N	N	10	-	18	A	N	N	N	N	15	-	18	N
15	UN	UN	UN	N	11	+	15	A	N	N	N	N	14	-	20	N
16	N	N	N	N	12	-	18	N	N	N	N	N	12	-	15	N
17	A	UN	UN	N	14	+	20	A	N	N	N	N	15	-	17	N
18	A	UN	UN	N	11	+	17	A	A	A	A	N	14	-	20	P
19	A	UN	UN	N	12	+	16	A	N	N	N	N	16	-	18	N

A: *Absent,* ***N:*** *Normal,* ***P:*** *Partial.* ***UN:*** *Uncertain,* ***MR:*** *Menace Response,* ***DR:*** *Dazzle Reflex,* ***PLR:*** *Pupillary Light Reflex,* ***PR:*** *Palpebral Reflex,* ***IP:*** *Intraocular Pressure,* ***FD:*** *Fluorescein Dye,* ***STT:*** *Schirmer Tear Test,* ***FE:*** *Fundus Evaluation*

mild in three eyes, moderate in five eyes, and severe in eleven eyes. Fluorescein staining on the graft was positive in most of the cases (10/19 eyes, 52.63%). The epithelization was monitored to have started 10 days after the operation and continued until the 3rd week. The existing corneal edema around the cornea and the suture line was maintained until 21-30 days of the surgical procedure. The menace reflex and pupillary light reflex could not be precisely evaluated due to the corneal edema and vascularization, particularly in the cases with central and large lesions (6/19 eyes, 31.57%), and fundus examination could not be performed (16/19 eyes, 84.21%).

An inflammatory reaction with superficial inflammatory granulomas around the sutures and the proliferation of the granulation tissue on the PRF membrane developed within the postoperative two weeks *(Fig.7-A,B)*. Corneal edema and vascularization were still present yet decreased,

Fig 8. A- The appearance of the 5th case 1 month after the operation and mild corneal opacity, **B-** Three months postoperative view of the left eye of cat 3 showing moderate corneal scar, **C-** Note severely opaque cornea

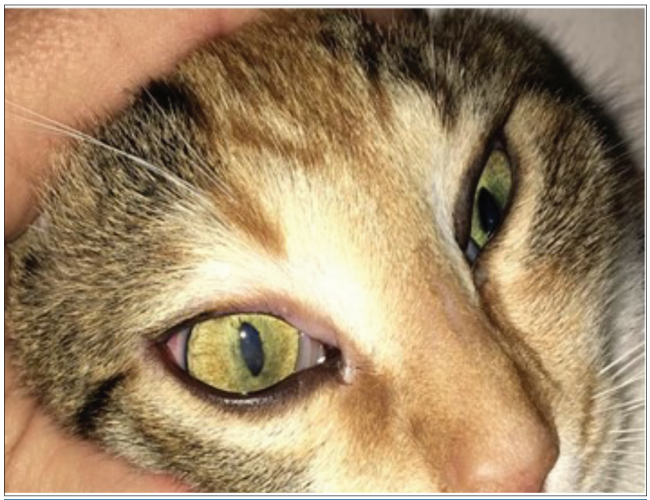

Fig 9. Ninety-days postoperative, appearance of the left eye

and fluorescein staining was negative except for ten cases which showed a minor epithelial defect on the graft. The menace response was present even though the PRF membrane trans-plantation site and surrounding corneas were edematous (13/19 eyes, 68.42%). Other ophthalmological examinations, apart from the fundus evaluation, could be performed, and the visual status of the patients, which could not be determined before the operation, could be more easily evaluated *(Table 3)*.

The complete integration of the PRF membrane graft with re-epithelialization and moderate corneal neovascularization was present in all eyes three weeks after surgery *(Fig. 8-A,B,C; Fig. 9)*. In the following weeks, the corneal cleaning phase was observed, in which epithelialization was completed in the cornea, and pre-existing corneal vascularization and granulation tissue began to be absorbed. Fluorescein staining negative in all cases. Corneal edema and vascularization next to the suture line decreased significantly and even completely disappeared in some cases. Fundus examination was successfully performed after the 3rd postoperative week in all cases, in which it failed to be performed, and preoperative vision could not be determined (17/19 eyes, 89.47%). The thinness of the wound site of the cornea increased significantly at 10, 14, 21 and 30 days postoperatively compared to pre-operatively.

Long-Term Follow-up: Up to 8 Months Postoperative Clinical Findings

At the 60th day-ophthalmic follow-up control, the morpho-anatomical outcome was favorable, and visual function was observed in all eyes; however, visual function status was relatively low in 2/19 eyes. Good cosmetic and visual results were provided in a very short time. Fundus was fully visible in 17/19 eyes, while fundoscopy could be partially performed in 2/19 eyes due to corneal opacity and anterior synechia sequelae *(Table 3)*. The eyeball integrity was maintained, and eyeball loss was prevented in both cases. However, there was limited vision due to the extended central corneal opacity even after the postoperative medical treatment (as assessed by threat responses performed at various visual fields). The degree of corneal opacity was severe in two eyes (10.5%), moderate in another two eyes (10.5%), blurry in 8 eyes (42.1%), and there was complete transparency in six eyes (31.5%). No improvement was observed in the wound appearance of the patients during the postoperative 1-8 months. The success of in this study was defined as complete corneal repair and integrity (100%) and the presence of significant visual acuity at final re-assessment (89.4%). Corneal defects were repaired in all patients, and a good vision was achieved in seventeen (89.4%) of 19 patients.

Discussion

Deep corneal ulcers associated with descemetoceles are noteworthy ocular diseases, with serious complications, such as sudden corneal perforation and eye loss requiring urgent intervention [6]. Aggressive treatment is required to minimize these complications, stop the progression of the lesion, which can be ruptured at any time, prevent the formation of corneal perforation, repair the thin cornea, and allow it to heal. Providing integrity and transparency for the cornea should be provided with surgical treatment [17]. Ideally, since many corneal ulcers affect the central or paracentral cornea, the surgical outcomes followed should be not only tectonic but also visual, and corneal opacities that may interfere with the patient's vision should be avoided [6,16]. In recent years, there has been growing interest in regenerative medicine, which aims to repair, replace and renew damaged tissues [18]. In the current study, we aimed to use and evaluate the PRF membrane, which

is accepted as a new method in humans, in veterinary ophthalmology as an alternative option with low biological risk and easy access to ocular surface reconstruction.

In our study, the PRF membrane, a new (second)-generation, autologous, thrombocyte concentrate developed by Choukroun et al.[12] for jaw-facial surgery applications, repaired deep defects. This grafting method used in hard and soft tissue surgery was initially tested by Alio et al.[18] in corneal perforation in humans and then by Can et al.[5] in descemetocele cases. The PRF membrane transplantation generated successful results in their use in human corneal reconstruction due to the membrane's anti-inflammatory properties and minimal scarring [5]. In addition to the usual phenomena, the authors noted that the membrane could be obtained relatively quickly, was easy to apply, cost-effective, and provided good corneal integration [5]. Its use in descemetocele cases in humans led us to consider applying it to cats due to its roughly similar composition [5]. The presented study aimed to confirm its usefulness in corneal reconstruction in felines considering other reconstruction techniques that have been confirmed for use in the treatment of deep ulcers in veterinary ophthalmology. In this study, autologous PRF membrane transplantation was performed in 19 eyes of 19 cats in the surgical treatment of descemetocele-related deep corneal ulcers. Before the preparation of the graft, necrotic, infected corneal tissues required complete ablation. Ablation of these lesions was performed systematically to enable the graft's positioning and accelerate and facilitate the operation. The graft was larger in size than the diameter of the corneal defect, which provided a stable position in the defect and minimized the risk of tearing the biomaterial when suturing. The fibrin transformed into a membrane graft is Alio et al.[18] and Can et al.[5] planted on a healthy cornea as described.

The PRF membrane graft is effective in stabilizing a markedly worsening disease process. The membrane clinically formed by a clot is cheap, easy-to-use, and mechanically robust, and its elasticity facilitates and maintains the stability of the surgical suture application [5]. These biomaterials, which are used as an alternative to bone grafts in humans as clot plugs in cavities, graft material at the implantation sites, and bone defects, can be sutured to the wound in the lesions with soft tissue deficiency, enabling increased tissue consistency. Based on the indication, three different surgical techniques can be used for PRF membrane transplantation: inlay, overlay, and combined [5,19]. Shukla et al.[20] compared the results of these three techniques in myringoplasty in humans and achieved higher success rates in the combined method. Tayşi et al.[21] deduced that the double-layered PRF membrane is a better barrier to bone healing than the single-layer membrane, and the monolayer membrane does not provide sufficient stabilization. In some recent reports in which this biomaterial was used for human corneal reconstruction, the PRF technology revealed biological properties similar to the amniotic membrane (AM), with its regenerative, anti-inflammatory, antifibrotic and anti-microbial properties. It has been stated that the PRF can be used as an inlay-graft, overlay-patch, or in the combined techniques like the AM application previously mentioned for descemetoceles for similar conditions depending on the lesion's size and depth [22]. In the inlay technique, the PRF membrane can be applied as a single or double layer. A single-layer graft can be placed without suturing with AM and therapeutic bandage contact lens (TBCL). In the multilayer membrane application, the thicker PRF membrane is placed directly onto the descemetocele area to fill the stromal defect (similar to AM's inlay application). A thinner membrane is placed on top to cover the cornea (similar to AM's overlay application). Likewise, Sanchez-Avila et al.[23] reported the usage of fibrin membrane alone in combination with membranes such as AM or Tutopatch, a collagen patch derived from the bovine pericardium. While investigating the effect of AM on corneal ulcers, which is reported to be structurally similar to the PRF membrane, Khokhar et al.[24] suggested the use of multilayer AM for the treatment of deep corneal ulcers. Based on this, Can et al.[5] applied the double-layer technique by placing a deliberately thicker PRF membrane filling the stromal ulcer, similar to the inlay-graft technique previously used for AM in the descemetocele area, and placing the thinner membrane covering a particular corneal area on top of the first following the overlay-patch technique described for AM. In the present report, a single-layer sutureless PRF membrane graft (inlay graft) with TBCL was also applied in descemetocele cases. The PRF membrane graft was conveniently placed as a filling in the descemetocele area and served as a scaffold for the defect and epithelial cells. Alio et al.[18] simultaneously used the PRF membrane and the clot. The patch's fibrin threads contribute to the defect's filling by binding to the clot and the stromal collagen fibers of the cornea. Ali et al.[18] suggested that the combined use of autologous fibrin membrane and E-PRP clot is a safe and effective alternative for the repair of corneal perforations. In our study, we applied a thicker PRF graft adjusted to the size of the defect by the inlay technique within the borders of the ulcerated corneal area to support the descemetocele and compensate for the stromal loss. Then, we utilized the onlay-patch technique by applying the thinner second layer on top to cover the ulcerated area and the graft and provide a scaffold for the epithelial cells, facilitating the underlying epithelium's healing. Applying a double layer PRF membrane was noted to have stabilized the course of the disorder in these lesions.

Kim et al.[25] indicated that application of biomaterials such as AM, equine renal capsule, equine pericardium,

porcine small intestinal submucosa (PSIS), and porcine urinary bladder graft (UBM) targeted to sustain moisture, avoid dehydration, and provide structural support to scar tissue during nictitating membrane flapping in managing various corneal disorders such as deep corneal ulceration, corneal perforation, corneal sequestrum, and descemotecele. In addition, it has been reported that the combination of AM and NMF application accelerates healing in corneal reconstruction in dogs [16]. Chow and Westermeyer [11] reported that, like a contact lens applied in human patients, the NMF provides mechanical protection to the healing cornea against trauma and eyelid friction. An NMF was temporarily fixed to the upper eyelid for 15 days by Laguna et al.[26] to protect the frozen lamellar corneal graft from flashing movements and help maintain pressure on the graft surface; for 18 days to protect the UBM graft by Balland et al.[10]; for three weeks to prevent drying and shedding of the porcine SIS graft by Goulle [27]. Alio et al.[18] suggested a 7-day-long partial tarsorrhaphy instead of NMF followed by the PRF membrane application to restrict eyelid movements, enable wound healing, and stabilize the fibrin membrane and clotting adhered to the ocular surface, while Can et al.[5] preferred a 10-day-long TBCL application, supporting the PRF membrane to avoid corneal perforation. We performed a 10-day-long NMF fixation instead of applying contact lenses, followed by the double-layer PRF membrane technique. The NMF membrane applied to 19 eyes was removed to examine graft integration, corneal integrity, and corneal infection status after a follow-up of ten days. We showed that NMF application protected the PRF membrane and prevented dehydration. Complications such as premature loss of biomaterial, drying, and graft failure were experienced in none of the cases, and ten days were considered sufficient for the application period.

There are three crucial stages in the PRF membrane's integration into the cornea: corneal neovascularization and proliferation of epithelial and stromal tissue, producing corneal transparency and maintaining corneal integrity, and remodeling the extracellular matrix (ECM) [5,28]. Corneal neovascularization is a crucial phase of corneal healing and the first step of PRF in the corneal stroma. Providing nutrition for corneal cells in different pathological conditions is a reaction that occurs to support the healing process [19]. PRF membrane-induced corneal neovascularization and epithelization in human descemetocele cases are prominent within 10-14 days [11,18]. In our study, corneal vascularization was observed in all cases during the healing process, and it increased gradually from the tenth to the 14th postoperative day. Epithelialization was completed quickly within the same period as reported in previous studies [5,11]. Thus, the cornea regained its former transparent structure, and epithelization was generally completed by the third week.

This corneal neovascularization, which is part of the desired healing response, is almost always present at the time of corneal ulceration and is induced by surgery. Chow and Westermeyer [11] applied Acell Vet in corneal reconstruction in cats and dogs. After the graft was epithelialized or entirely vascularized, they used dexamethasone and cyclosporine topically for 2-3 months, until the desired improvement has been achieved, to stimulate regression of corneal vessels and reduce corneal fibrosis. Dulaurent et al.[29] used topical dexamethasone for dogs and topical indomethacin for cats for two weeks to minimize remaining corneal vessels once the epithelial barrier has been restored following a bovine pericardium graft application. Nevertheless, even if topical steroids maintain corneal clarity, they may cause other serious problems, as was recently reported in several Persian cats with corneal sequestra. Steroids can increase the lytic effect of corneal collagenase and are contraindicated in feline herpes virus infection [26]. They should be administered cautiously as suspensions to reduce corneal neovascularization, excessive granulation tissue, and scarring, except for cats suspected of a herpes virus infection. Goulle [27] suggests that topical dexamethasone should be administered with caution, usually for one to four weeks, depending on the intensity of scarring and corneal neovascularization. Thus, they administered dexamethasone initially once a day or every other day for about two weeks and then twice a day in some cases. However, cats treated with topical corticosteroids may be at risk of developing corneal ulcers due to reactivation of the latent herpes virus since up to 50% of asymptomatic cats have FHV1 DNA in the cornea [27]. Likewise, after the epithelialization of the PRF graft was completed, we administered topical steroidal drugs with care to reduce postoperative corneal vascularization, inflammation, formation of granulation tissue, and pain and to manage visual acuity, depending on the severity of corneal neovascularization and fibrosis. Unlike the previously reported data, no signs of infection were observed even in patients with suspected FHV-1. Likewise, no recurrence was noted during the follow-up period and steroid application.

Postoperative corneal clarity is achieved by the absence of blood vessels, pigmentation, and the regular arrangement of collagen fibrils in the stroma [30]. Serafini et al.[31] reported that type 3 collagen was synthesized at the lesion site during the normal stromal healing process, and this structure was relatively less regular, emerging as corneal scarring, potentially rendering visual impairment. The clinical reports indicate that the PRF membrane sutured to the corneal ulcer site provides corneal repair, similar to that obtained with conjunctival autografts, yet with fewer postoperative complications [5,18]. It was observed that the PRF membrane was completely dissolved, merging into the host cornea, and no permanent abnormal tissue islands

were noted, yet the corneal tissue was prone to scarring [32]. The results were reported to be quite successful in two different reports concerning the PRF membrane graft applications in corneal reconstruction in humans [5]. Our study revealed that epithelialization started on day ten and was completed in the third week. Satisfactory transparency with no recurrence was achieved in the lesion area, followed by the fibrovascular invasion of the membrane during the 3-month-follow-up period. Corneal edema was detected in almost all cats until the 21st or 25th to 30th days postoperatively, and then it started to fade. Corneal defects were repaired, corneal integrity and vision were restored in all 19 patients; however, the healing process was relatively limited in two patients. Compared to other surgical techniques recommended for descemetoceles in cats in the literature, PRF can be considered among the techniques with a quite successful outcome (89.4%) [18]. Furthermore, none of the cases showed bacterial contamination after the PRF membrane application in our study.

We deduced that the PRF membrane graft is a reproducible, cost-effective eligible biomaterial compared to other corneal grafts rendering a rapid application, and it provides favorable morphoanatomical and visual outcomes with relatively less scarring in the patients. It has been very effective in the early migration of vessels to the ulcerated area, which is crucial in repairing severe stromal loss, such as descemetocele, requiring urgent surgical treatment and thus accelerating healing. It provided good tectonic support during the healing process and eliminated the risk of perforation in ulcers in which the corneal tissue was significantly thinned.

In conclusion, the PRF membrane grafting successfully managed deep corneal ulcers in cats (89.4%), emerging as an excellent alternative to other corneal reconstruction methods that often cause severe corneal scarring and visual defects.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the corresponding author A. Demir).

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Competing Interests

The authors declare no conflict of interest regarding this report.

Author Contributions

AD and DOE made the design of the study. AD made the writing of the article, the treatment and controls, and the revision of the manuscript. YA and ZTS provided data collection, article writing, revision of the article draft, submission of the article and entry of subsequent revisions into the system.

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Research Article

Autologous Platelet Rich Plasma Have Positive Effect on Ram Spermatozoa During Cryopreservation in Non-Breeding Season

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Abstract: The aim of this study was to evaluate the effects of autologous platelet-rich plasma (PRP) supplemented egg yolk based extender on ram semen after thawing. Semen samples were collected from nine Kıvırcık rams and each semen was divided into four equal aliquots and mixed with different concentrations of PRP supported extenders [no PRP (control), 12.5x10^6/mL PRP, 25x10^6/mL PRP, or 50x10^6/mL PRP)]. Motility, plasma membrane functional integrity, acrosome integrity, mitochondrial membrane potential, DNA integrity and malondialdehyde concentrations (MDA) were measured and analyzed after thawing. The results showed that 25x10^6/mL PRP group had positive effect on motility (61.67±3.81), membrane functional integrity (71.00±2.96) and MDA levels (5.13±0.64) at post-thawed (P<0.05). It was determined that 25x10^6/mL PRP and 50x10^6/mL PRP groups were more effective than other groups in terms of mitochondrial membrane potential (69.50±1.93), acrosome integrity (78.04±2.65) and DNA integrity (5.33±0.92). The results of the study showed that autologous PRP has a protective effect in the cryopreservation of ram spermatozoa.

Keywords: *Ram semen, Platelet-rich plasma, Cryopreservation, Post-thawed*

Otolog Trombositten Zengin Plazma Üreme Mevsimi Dışında Koç Spermatozoalarının Kriyoprezervasyonu Üzerinde Olumlu Etkiye Sahiptir

Öz: Bu çalışmanın amacı, otolog trombositten zengin plazma (PRP) ilave edilmiş yumurta sarılı sulandırıcılar eritme sonrası koç sperması üzerine etkilerini değerlendirmektir. Dokuz adet kıvırcık koçtan elde edilen sperma numuneleri dört eşit hacme bölündü ve farklı konsantrasyonlarda PRP içeren sulandırıcılar [PRP yok (Kontrol), 12.5x10^6/mL PRP, 25x10^6/mL PRP ve 50x10^6/mL PRP) ile sulandırıldı. Çözdürme sonrası motilite, plazma membranı fonsiyonel bütünlüğü, akrozomal bütünlük, mitokondriyal fonksiyon, DNA bütünlüğü ve malondialdehit konsantrasyonu (MDA) ölçüldü ve analiz edildi. Sonuç olarak, 25x10^6/mL PRP içeren sulandırcının çözdürme sonrası motilite (61.67±3.81), plazma membran fonksiyonel bütünlüğü (71.00±2.96), ve MDA seviyeleri (5.13±0.64) üzerine olumlu etkisi olduğunu gösterdi (P<0.05). 25x10^6/mL PRP ve 50x10^6/mL PRP gruplarının mitokondriyal fonksiyon (69.50±1.93), akrozomal bütünlüğü (78.04±2.65) ve DNA bütünlüğü (5.33±0.92) açısından diğer gruplara göre daha etkili olduğu belirlendi. Çalışmanın sonuçları, otolog PRP'nin koç spermasının dondurularak saklanması üzerine koruyucu bir etkiye sahip olduğu görüldü.

Anahtar sözcükler: *Koç sperması, Trombositten zengin plazma, Kriyoprezervasyon, Eritme sonrası*

Introduction

Cryopreservation that enables the maintenance of the biological function of spermatozoa for a long time has been widely used in various species (mammals, insects, avians, etc.). However, it has detrimental effects on spermatozoa because of the intracellular ice crystallization, osmotic changes, cold shock, oxidative stress and reactive oxygen species (ROS). Lipid peroxidation in the cytoplasm membrane of spermatozoon causes ROS production during cryopreservation. Free radicals lead to stress on the spermatozoa membranes therefore, viability, motility,

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and fertilizing ability of spermatozoon decrease during the freeze-thawed process. In recent years, various substances have been supplemented to the semen extenders against lipid peroxidation and to improve sperm quality at post-thaw [1-3].

Platelet-rich plasma (PRP) is a plasma component that contains 3-7 times a higher concentration of thrombocytes than the physiological concentration of whole blood [3-6]. Additionally, it contains hyperphysiological growth factors (GF) (insulin-like GF I, II, epidermal (GF), connective tissue (GF), platelet derived (GF), nerve (GF), vascular endothelial (GF), hepatocyte (GF), interleukin 8 (IL8), fibroblast (GF) and transforming (GF)], histamine, serotonin, calcium ions, zinc ions, superoxide dismutase (SOD) and adenosine triphosphate (ATP) [3,7,8]. It is used successfully in reproduction, dermatology, and orthopedics in mammals because of these important factors. In addition, many of these factors (VEGF, TGF, IGF-1, NGF, zinc ions, ATP, SOD and platelet-activating factor) have positively effect on sperm motility, viability, mitochondrial function, and DNA integrity at post thawed [3,9-12].

The effects of PRP based extenders have been examined on the outcomes of routine spermatological evaluations (motility, viability, acrosomal integrity etc.) [3]. But previous reports have not focused on the effects of PRP on ram semen cryopreservation. Therefore, the aim of the current study was to evaluate the effect of various concentrations of autologous PRP on the quality of ram spermatozoa at post-thawed.

Material and Methods

Ethical Approval

The study was approved by Scientific Ethical Committee (Bursa Uludag University, Türkiye) (No: 2021-04/03).

Chemicals

Chemicals were purchased from Merck (Darmstadt Germany) and Sigma (St. Louis, MO, USA) in the study.

Experimental Design

This study was designed to determine the effectiveness of PRP supplemented to the semen extender on ram semen cryopreservation. For this intention, diverse concentrations of autologous PRP [no PRP (control), 12.5x10^6/mL PRP, 25x10^6/mL PRP, or 50x10^6/mL PRP)] were used in the extenders during non-breeding season. Each experiment was replicated five times throughout the study.

Semen Extender Preparation

Extenders contained 223.7 mmol/L Tris, 66.6 mmol/L citric acid, 55.5 mmol/L fructose, 4.03 mmol/L EDTA, 4 g/L penicillin G, 100.4 mmol/L trehalose, 3 g/L dihydrostreptomycin, 20% egg yolk in distilled water. Autologous PRP was supplemented to each group according to the experimental design [13].

For PRP preparation, commercial PRP kits (S&M PRP Unique STR Kits) were used. Briefly, PRP was obtained from each ram's blood sample 1 h before blood draw. Ram semen was collected five times every other day and PRP was prepared again in each application. 10 mL blood were collected from each ram and centrifugation (15 min at 3200 rpm) was made using a tabletop cold centrifuge device. The extraction of PRP was completed based on the method outlined in the commercially available separation system. From each ram, 800x10^6 - 920x10^6/mL autologous PRP platelet were obtained. The obtained PRP was activated by 10% calcium chloride. PRP concentrations were detected by a fully automatic five-type blood cell analyzer (Sysmex XT-1800i).

Semen Collection and Dilution

Nine Kıvırcık rams maintained with the same state of affairs were used for semen collection. Semen was collected with electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand). Collected ejaculates were transferred to the laboratory in a water bath (37°C). Only ejaculates which have rapid wave (> +3 on 0-5 scale), >75% motility and >1.0x10^9 spermatozoa/mL were used.

Briefly, each ram ejaculate was split into four equal aliquots and diluted (37°C) to a final concentration of almost 150x10^6 (spermatozoon/mL) with PRP supplemented extenders and control extender. Diluted semen was gradually cooled to 4°C and then equilibrated for 2-h at 4°C.

Semen Freezing and Thawing

After the equilibration, each diluted semen was loaded into 0.25 mL straws. Cryopreservation and thawing procedures were performed by the method of Alcay et al.[14]. According to this method, straws were frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C using the Nicool Plus PC freezing machine (Air Liquide, France). Then, the sperm-filled pipettes were dipped in liquid nitrogen and left in the liquid nitrogen container until examined.

Semen Evaluation

After thawing, sperm motility, plasma membrane integrity (hyposmotic swelling test (HOST)), capacitation status (Chlortetracyclin (CTC) staining), mitochondrial activity (R123; Invitrogen TM, Eugene, OR, USA) and DNA integrity (terminal deoxynucleotidyl transferase) using -mediated dUTP pseudo-end labeling (TUNEL)) was evaluated. All measurements and evaluations made from the beginning to the end of the study were made by the same person.

- Motility

Ram sperm motility assessment was performed using a phase-contrast microscope at 400x magnification (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) with the slide heated to 37°C.

- Membrane Functionality

For the assessment of the plasma membrane functional integrity, the hypo-osmotic swelling test method is used which was previously applied by Alcay et al.[14]. Following this method, the membrane integrity of the ram sperm was evaluated by observing the frizzled tails.

- Acrosome Integrity

For this evaluation method, a sample of 10 µL spermatozoa was added in 100 mL of PBS and then centrifuged for 5 min. The sperm pellet obtained after centrifugation was resuspended again in 100 mL PBS and smeared on the slide. Smeared-slides were left in acetone fixation at 4°C for 15 min. After fixation, smears were stained with FITC-PSA solution for 1 h at 37°C. After the staining process was completed, at least 200 ram spermatozoa were evaluated under a fluorescent attachment microscope [15].

- Mitochondrial Activity

Mitochondrial functions were assessed with fluorescent stains, PI, and Rhodamine (R123) combination [16]. Semen samples were first washed with phosphate-buffered saline and then centrifuged at 4000 g, 30 s. PBS was added to dilute the sperm sample (1/10 mL). Rh123 was then added to a final concentration of 5 µg/mL, and the sample incubated for 5 min at 37°C in the dark. At least 200 spermatozoa were used and the results were expressed as a percentage (%).

- DNA fragmentation

DNA fragmentation rates were evaluated by the TUNEL technique using *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim Germany) according to the manufacturer's protocol with slight modifications [17]. In brief, one drop of resuspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min. The slides were washed in PBS and stored at 4°C until use. The permeabilized slides were incubated in the dark chamber at 37°C for 1 h with the TUNEL reaction mixture which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. TUNEL positive sperm cells. Each microscopic field was evaluated first under fluorescence microscopy (400× magnification) to determine the number of reactive sperm and then under phase-contrast microscope to determine the total number of sperm per field.

- Malondialdehyde (MDA) concentrations

To assess the MDA concentrations the method of Sharafi et al.[18] was performed. Briefly, 0.25 mL of diluted semen sample was treated with 0.25 mL of cold 20% (w/v) trichloroacetic acid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 min in a 100°C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using the Spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/mL.

Statistical Analysis

Data obtained from this study are presented as mean ± standard deviation. Shapiro Wilk test was used for the normality test. The mean values of the obtained parameters were analyzed using the Kruskal Wallis test, which is an ordered one-way analysis of variance test. Statistical differences between the means of the treatment groups with differences were determined by the Mann Whitney U test. Differences with P values less than 0.05 were considered statistically significant. All analyzes were computerized using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA).

Results

In the study, the mean percentages of motility, plasma membrane functionality, acrosome integrity, mitochondrial function, and DNA fragmentation rates of nine fresh semen samples were 81.25±2.31, 88.87±2.85, 93.00±2.44, 89.75±3.49, and 4.25±0.46 respectively. Sperm quality was significantly reduced by the cryopreservation process compared with the fresh samples (P<0.05).

Table 1 shows the effects of different concentrations of PRP on ram sperm parameters at frozen-thawed. PRP25 and PRP50 groups significantly improved the motility, in comparison to the control and 12.5 groups without PRP addition at post-thawed (P<0.05). In addition, the highest percentage of motility rates were obtained from the PRP25 group (P<0.05). The percentages of plasma membrane functional integrity were higher in PRP groups compared to the control group (P<0.05). Also, the highest membrane integrity rate was obtained in the PRP25 group (P<0.05).

The higher acrosome integrity and mitochondrial function rates were obtained in the PRP groups compared to the control group (P<0.05). The highest DNA damaged spermatozoa were obtained control group compared to the PRP groups at post-thawed (P<0.05). As shown in *Table 2*, it was found that the MDA levels were lower in PRP25 group compared to the other groups (P<0.05).

Discussion

Platelet-rich plasma which has a source of growth factor has been widely used in regenerative medicine because of

Table 1. *The mean of studied ram sperm post-thawing parameters on different extender groups*

Variable	PRP Concentrations (x10⁶/mL)			
	0	**12.5**	**25**	**50**
Motility (%)	50.00±0.52[a]	52.60±0.82[a]	61.67±0.78[b]	56.88±1.20[c]
HOST (%)	60.46±0.68[a]	63.80±0.66[b]	71.00±0.60[c]	66.88±0.76[d]
Acrosomal Integrity (%)	73.00±0.42[a]	75.60±0.47[b]	78.04±0.54[c]	76.58±0.45[bc]
Mitochondrial function (%)	60.58±0.72[a]	64.64±0.61[b]	69.50±0.39[c]	66.42±0.83[bc]
DNA fragmentation (%)	9.75±0.20[a]	8.92±0.16[b]	5.33±0.19[c]	5.96±0.20[c]

Data is presented in Mean± S.E.M. Different letters within the same rows show significant differences among the groups (P<0.05)

Table 2. *Malondialdehiyde (MDA) levels in frozen-thawed drone sperm*

Groups	Control	PRP12.5	PRP25	PRP50
MDA (nmol/mL)	7.75±0.41[a]	7.00±0.27[a]	5.13±0.23[b]	5.63±0.18[c]

Data is presented in Mean± S.E.M. Different superscripts (a,b,c,b) in the same line indicate significant differences (P<0.05)

its cytoprotective properties. In the current study, we have investigated the effect of autologous PRP supplemented extenders on the quality of ram spermatozoa at post-thawed.

Cryopreservation ensures that genetic materials are preserved for a long time. However, it is known that the freezing-thawing process has undesirable effects on the fertilization ability of spermatozoa. These negative effects decrease motility, viability, plasma membrane, and acrosome integrities of spermatozoa. In the study, ram sperm have been negatively affected by the cryopreservation process compared to fresh spermatozoa (P<0.05).

Motility provides useful information concerning spermatozoon quality and oocyte penetration ability [19]. The cryopreservation process damages the cell membrane structure and inhibits the production of ATP, which ultimately leads to a reduction in sperm motility. PRP contains multiple biologically active ingredients which responsible for sperm motility and viability. In this study, we have shown that the presence of autologous PRP concentrations in the extenders increased ram spermatozoon motility compared to the control group at post-thawed (P<0.05). In the studies, the motility rates of ram spermatozoon cryopreserved with various commercial or non-commercial extenders ranged between 25% - 62% [13,20-24] at post-thawed. Our study shows that post-thawed motility values in high dose PRP groups (PRP25 and PRP50) were in good agreement with the findings of these studies. In our study, although rainbow trout seminal plasma (RTS) supplementation caused a clear increase in motility, the PRP12.5 group had not sufficient effect to make a statistical difference compared with the control group at post-thawed. When the PRP doses were compared among each other, the PRP25 group caused an increase in motility.

The functionality of the plasma membrane that is essential for spermatozoon metabolism plays a crucial role in the oocyte fusion of spermatozoon [25]. However, coldshock and lipid peroxidation have negatively affect membrane permeability and integrity during cryopreservation [26]. Therefore, it is crucial to keep integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality [14]. In the study, the plasma membrane functional integrity in the PRP25 group was higher than in the other groups (P<0.05). The HOST values are in agreement with the earlier researches [13,14,20].

Acrosome examination is an important method that determines the fertilization ability of spermatozoa [27]. During the cryopreservation process, acrosome integrity and fertilization ability deteriorate. However, PRP groups successfully protected acrosome integrity during the cryopreservation process. These results are in agreement with the previous researches [23,28,29].

Sperm needs the energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways [30]. Mitochondrial membrane potential is evaluated as a parameter related to the production of ATP by the spermatozoon mitochondria through oxidative phosphorylation and capacitation. Low mitochondrial membrane potential may occur due to spermatozoa anomaly or cryo damage during freeze-thawed process [31]. Therefore, it is important to determine the potential of the mitochondrial membrane for sperm quality [32,33]. In the study, mitochondrial function was better preserved in PRP groups compared to the control group (P<0.05). Similar results were obtained in previous research [30,34-36].

Sperm DNA integrity is important not only for the successful transfer of genetic material to future generations but

also for proper fertilization, quality embryo development and pregnancy. Protecting the integrity of DNA during cryopreservation also has great importance not to disrupt the early development of the embryo [27]. IGF-1 and NGF which were ingredients in PRP have been proven to improve DNA integrity. In this study, it was observed that PRP groups were resistant to the cryopreservation process. A similar result was obtained in previous research [3].

Oxidative damage is one of the most common cryopreservation damages and it may be evaluated by MDA levels which is a key product of polyunsaturated fatty acid's peroxidation in the cells [37,38]. PRP has an antioxidant effect in mammalian cells therefore it has also a positive effect on sperm during cryopreservation. In our study, the lower MDA levels were obtained PRP25 and PRP50 groups (P>0.05).

Considering all sperm parameters; autologous PRP has a protective effect on cryopreservation of ram spermatozoa. The PRP25 group was the optimum for semen preservation. Future studies might be focusing on PRP supplementation to evaluate reproductive success when used to fertilize the sheep.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the corresponding author (S. Alçay).

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Conflict of Interest

The authors declare that there were no conflicts of interest in the realisation of this research.

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Author Contributions

SA, MA, HS designed the experiment. SA, AA, DK, performed the experiment. AA, MY, MK analyzed the data. SA, AA made tables, and wrote the paper. All authors reviewed and approved the final manuscript.

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RESEARCH ARTICLE

Blood and Milk Beta-hydroxybutyric Acid Concentrations in Different Dairy Cattle Breeds and Association of Subclinical Ketosis with Postpartum Health Disorders, Culling Rate, Body Condition Score, Parity and Milk Production in Holstein

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Abstract: The aim of the study was to analyse beta-hydroxybutyric acid concentration in the blood (BBAC) and milk (MBAC) at postpartum week 2 (PPW2) and week 4 (PPW4) in Holstein (n=216, 8 farms), Montbeliard (n=23), Simmental (n=38) and Holstein/Montbeliard Crossbreed (n=23, BBAC only). Furthermore, the prevalence of subclinical ketosis (SCK) and its association with postpartum health disorders (PPHD), body condition score (BCS) and average daily milk yield in 90 days in milk (90DIM) were evaluated in Holstein. Holstein-Crossbreed and Montbeliard had significantly lower postpartum BBAC than others. Primiparous Montbeliard and Holstein had significantly higher MBAC at PPW2 than PPW4. Cows having BCS2 and 4 at calving had higher MBAC at PPW2 and 4 that was associated significantly with metritis and multiple diseases. Holstein with BCS4 at calving had higher BBAC at PPW2 and 4. SCK prevalence in the blood (BBAC≥1.2 mmol/L, BSCK) and milk (MBAC=100 µmol/L, MSCK1), (MBAC≥200 µmol/L, MSCK2) and (MBAC≥100 µmol/L, MSCK1/2) was 8.3, 11.8, 5.8 and 17.3% at PPW2 and 4.7, 4.9, 6.9 and 11.9% at PPW4 in Holstein respectively. Holstein with SCK was more likely to develop PPHD in 90DIM. MSCK1 and MSCK1/2 did not associate milk production loss in Holstein. Holstein with BSCK and MSCK2 at PPW2 had a 6.7 kg loss of average daily milk yield in 90DIM. Culling rate was 3.7% in Holstein and MSCK2 positive Holstein at PPW2 was significantly more likely to be culled (25%, Odds:11.2, P<0.05) in 90DIM. In conclusion, cows having BCS2 and 4 at calving had higher MBAC and BBAC at PPW2 and 4. Holstein-Crossbreed and Montbeliard had much lower postpartum BBAC than Holstein and Simmental. BSCK and MSCK2 caused significantly risk factor for PPHD, culling rate and milk production in Holstein.

Keywords: *Beta-hydroxybutyric acid, Holstein, Metabolic diseases, Montbeliard, Simmental, Subclinical ketosis*

Farklı Süt Sığırı Irklarında Kan ve Süt Beta-hidroksibütirik Asit Düzeyleri ve Holstein'larda Subklinik Ketozisin Postpartum Hastalıklar, Sürüden Ayırma, Vücut Kondisyon Skoru, Parite ve Süt Verimi İle İlişkisi

Öz: Bu çalışmanın amacı beta-hidroksibütirik asit konsantrasyonlarını kanda (KBAK) ve sütte (SBAK) Holstein (n=216, 8 çiftlik), Montbeliard (n=23), Simmental (n=38) ve Holstein- Montbeliard melezlerinde (HMM, n=23, yalnız KBAK) postpartum 2. ve 4. haftada (PPH2 ve PPH4) ölçmektir. Ayrıca, subklinik ketozis (SK) prevalansı ile irtibatlı doğum sonrası ilk 90 gündeki postpartum metabolik hastalıklar (PPMH), vücut kondisyon skoru (VKS) ve günlük ortalama süt verimi Holstein ırkında değerlendirilmiştir. HMM ve Montbeliard'larda diğerlerine göre anlamlı olarak daha düşük KBAK oranı tespit edilmiştir. Primiparous Montbeliard ve Holstein ırklarında SBAK PPH2'de PPH4'e göre önemli oranda yüksek çıkmıştır. VKS'si doğumda 2 veya 4 olan hayvanlar PPH2 ve 4'de anlamlı derecede daha yüksek SBAK'a sahip oldukları görülmüştür ve bu hayvanlarda metritis ve çoklu hastalık görülme oranı yüksek çıkmıştır. Doğumda VKS4'e sahip Holstein'lar PPH2 ve 4'te daha yüksek KBAK'a sahiplerdi. Holstein'larda SK prevalansı PPH2'de kanda (KBAK≥1.2 mmol/L, KSK) ve sütte [SBAK=100 µmol/L (SSK1), SBAK≥200 µmol/L (SSK2), SBAK≥100 µmol/L (SSK1/2)] sırasıyla 8.3, 11.8, 5.8 ve 17.3% ve PPH4'de sırasıyla 4.7, 4.9, 6.9 ve 11.9% olarak tespit edilmiştir. SK pozitif olan Holstein ırkında, doğum sonrası ilk 90 günde PPMH gelişme oranı anlamlı derecede risk oluşturmuştur. SSK1'in ve SSK1/2'nin süt verimi ile ilişkisi tespit edilmemiştir. PPH2'de hem KSK hem de SSK2'ye sahip Holstein'larda, ilk 90 günde günlük ortalama 6.7 kg süt verimi kaybı görülmüştür. Doğum sonrası ilk 90 günde sürüden ayırma oranı Holstein ırkında %3.7 oranında görülmüş ve bunlardan PPH2'de SSK2 pozitif olanlar anlamlı derecede yüksek riskli çıkmışlardır (Odds 11.2, %25, P<0.05). Sonuç olarak, doğumda VKS2 ve 4'e sahip hayvanlarda PPH2 ve 4'te SBAK ve KBAK düzeylerinin daha yüksek olduğu gözlenmiştir. Montbeliard ve HMM'lerin postpartum KBAK'ları Holstein ve Simmental'lerden daha düşük çıkmıştır. KSK ve SSK2, Holstein'larda PPMH, sürüden ayırma ve süt verimi için önemli bir risk oluşturmuştur.

Anahtar sözcükler: *Beta-hidoksibütirik asit, Holstein, Metabolik hastalık, Montbeliard, Simmental, Subklinik ketozis*

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Introduction

Dairy cows must orchestrate the metabolic challenges during the transition from dry-period to early lactation to support milk production with an adequate glucose supply. These critical production stages can result in several postpartum metabolic disorders if dairy cows do not overcome the negative energy balance (NEB) due to reduced dry matter intake and other complications [1-3]. The NEB is the main reason during the transition period and can negatively affect milk production due to subclinical ketosis (SCK) [4], metabolic and reproduction parameters [3,5,6] and farm profitability through decreased milk production and increased risk of metabolic diseases [3,7-9]. Increased demand for milk consumption resulted in increased annual milk production per cow from roughly 2.000 kg to 10.300 kg worldwide [1]. The dairy cattle population transformed from indigenous low milk yielding breed to high milk yielding dairy breeds from 1991 to 2019 which resulted in increased milk production per cow from 1.4 ton to 3.1 ton and annually from 8.6 Mio ton to 20.7 Mio ton respectively in Turkey [10]. However, this brought problems of metabolic and reproductive diseases such as ketosis, displaced abomasum resulting in early culling [10]. SCK was associated with increased level of beta-hydroxybutyric acid (BHBA) also called hyperketonemia or hyperketolactia in the absence of clinical ketosis signs and it is a common disease for high milk yielding dairy cows. Beta-hydroxybutyric acid concentration (BAC) in the blood (BBAC) or milk (MBAC) is one of the most tested ketone bodies among others as such acetone and acetoacetic acid in recent years [3,9,11]. Testing of BBAC [9,11-13] and MBAC [14-16] indicate the NEB, that can result in clinical ketosis and SCK in early lactation. Thus, hyperketonemia became an economically relevant postpartum metabolic problem in terms of its impact on farm profitability, especially in Holstein dairy farming [3,7,8,17], however, there are not enough papers published about Montbeliard, Simmental and Holstein-Crossbreed. Various studies in Holstein revealed a prevalence of 21.8% [11] and 24% [13] worldwide if tested in the blood, in which a cut-off level for BBAC≥1.2 mmol/L was taken. Studies from Turkey reported that the prevalence was 11.2% [11] and 19.4% [12] with the same BAC threshold. Recent studies showed that checking of MBAC found large acceptance by using Fourier-Transform Infrared Spectrometry [16] or milk ketone strips [9,14]. A prevalence of 39% in Holstein was reported by using milk ketone strips in European countries. This rate was 22.6% by using Fourier-Transform Infrared in Canada [14]. The cut-off value of MBAC for the definition of SCK varied among the studies. Few papers used MBAC to define SCK prevalence. Ranges of MBAC to classify cows with suspect of hyperketolactia (0.15 to 0.19 mmol/L) or positive hyperketolactia (≥0.20 mmol/L) were reported by Benedet et al.[9] and others [14,18]. The relationship between BBAC and MBAC and milk production was studied in Holstein cows [15,19]. Most of the studies about the prevalance of SCK were cunducted via a blood test only. A few studies reported the correlation between BBAC and MBAC and the prevalence of SCK defined by different cut-off values in milk, as well as its association with PPHD, culling rate, body condition score (BCS) and parity. The majority of the studies about the prevalence of SCK and its association with PPHD were conducted in Holstein dairy farms. Montbeliard and Simmental were classified in the same family [20,21] and there are no comparative analysis about BBAC and MBAC in different parities compared to Holstein. Although there was a correlation between hyperketonemia and hyperketolactia [22], there is also a lack of research conducted for the comparison of BAC in the blood and milk in various breeds at most critical time points postpartum week 2 and 4 (PPW2 and PPW4) worldwide. The aim of the present study was to compare the relationship of BBAC and MBAC with parity and BCS at two different postpartum time points in Holstein, Simmental, Montbeliard and Holstein-Crossbreed dairies. Furthermore, the present study aimed to analyse the prevalence of SCK defined with hyperketonemia and hyperketolactia at two most important time points such as PPW2 and PPW4 and its associations with PPHD, culling, BCS, parity and milk production in Holstein.

Material and Methods

Ethical Statement

The study was approved by the Animal Experiments Local Ethics Committee of University of Erciyes (EÜHADYEK) with the ethical approval number of 15.05.2019/05/19-113.

Animals and Grouping

This is a randomized field study. The study was conducted in 4 provinces (İzmir, Aydın, Muğla and Denizli) of Turkey. The present study was conducted between September 2019 and March 2020. Three hundred lactating cows in 11 integrated dairy cattle farms consisting of Holstein (n=216, farm 1 to 8), Simmental (n=38, farm 9 and 11), Montbeliard (n=23, farm 1) and Holstein-Crossbreed (HC) (HolsteinxMontbeliard, n=23, farm 10) were enrolled for the study. Parity groups were created as primiparous (PRP) and multiparous (MUL) due to association of SCK with the parity [13,22]. Furthermore, groups were created for the definition of SCK in the blood (BSCK) and milk (MSCK1, MSCK2 and MSCK1/2) based on appropriate cut-points of BBAC and MBAC. Combined prevalence groups such as BSCK/MSCK positive and BSCK or MSCK positive both at postpartum week 2 and 4 were created to observe their effects on the average daily milk yield (ADMY) in Holstein. Farms (n=8) that had an automatic milking system, milk yield recording database, and complete milk

yield recording for study animals (n=206 Holstein) were enrolled in the milk production analysis.

Animal Feeding

All farms had a professional self-ration program and, cows were fed with a ration according to the production cycle, energy, mineral and other nutrients requirements (dry period, close-up, early lactation). Water was served *ad libitum*. Farm feeding strategy and ration have not been changed or specifically prepared for this study and throughout the research period. Cows in the study farms fed with a ration consisting of maize silage, hay, alfalfa, concentrated milk feed, maize flake, cottonseed, limestone, soy sauce, vitamin and mineral premix that had the metabolic energy of 41.77 to 54.30 Mcal/day for a cow in early lactation.

Beta-hydroxybutyric Acid Analysis and Definition of SCK in the Blood and Milk

BBAC was analysed in the individual whole blood samples collected from the coccygeal vein by a practical cow-side analyser [23] (Medtrust Wellionvet Belua, Med Trust Handelsges.m.b.H., Austria) at PPW2 and PPW4. Semi-quantitative MBAC was analysed at the same times like blood test in 50 mL of freshly taken individual milk samples (within 5 min after collection) with milk-test-strips [24] (Ketotest, Elanco). SCK without clinical signs of ketosis (e.g. constipation, anorexia, rumen dysfunction, reduced rumination) was defined by a cut-off point of BBAC≥1.2 mmol/L (BSCK) in the blood [10,13] and MBAC=100 µmol/L (MSCK1), MBAC≥200 µmol/L (MSCK2) and MBAC≥100 µmol/L (MSCK1/2) in the milk as recommended by the test kits manufacturer and others [9,14,15,18].

Body Condition Scores and Postpartum Health Checks

BCS controls were performed according to the recommendations by Edmonson et al.[25] based on a scale from 1 to 5 at calving (postpartum day 0), postpartum day 30 (PP30) and day 60 (PP60). Groups for BCS<2.5 (BCS1), BCS ≥2.5-<3.5 (BCS2), BCS≥3.5 to <4.0 (BCS3) and BCS ≥4.0 (BCS4) were set up. The difference of BCS relative to calving was the body condition loss or gain [26]. Cows were monitored and evaluated daily from the clinical health point of view for PPHD, any single or multiple diseases or culling were registered immediately in 90 days in milk (90DIM). They were specifically checked for retained placenta, displaced abomasum, metritis, mastitis, cystic ovarian, lameness, clinical ketosis, milk fever or combined multiple diseases (more than 1 disease) in 90DIM because they were most prevalent PPHD associated with SCK [3,5-8,22].

Milk Yield Recording

The daily milk yield of Holstein (n=206) was recorded automatically in the study farms (n=8) where various automated milking system was set and continuously recorded in a data base. Milk yield was taken directly from computerized farm database.

Statistical Analysis

Statistical analyses were performed using the SPSS (version 22) software and the results were evaluated for α=0.05. The normality of the data was evaluated by Kolmogorov-Smirnov and Shapiro-Wilks tests. Mann-Whitney-U, Wilcoxon Signed Ranks and Kruskal-Wallis, Friedman were used for statistical analysis because of the non-normality of the data and small sample sizes. Arithmetic mean (m), standard error (se) or minimum and maximum values were presented as descriptive statistics. Prevalence of BSCK and MSCK1, MSCK2 and MSCK1/2 was presented as numeric, positive, negative and % in Holstein. In order to evaluate the disease incidences and dependency of BHBA between PPW2 and PPW4, Fisher's exact test was used. Incidence of the PPHD was presented as a percentage. The odds ratio was determined for each of the diseases (for those with sufficient data for computation) in SCK groups. Pearson correlation coefficients were calculated between BBAC/BSCK and MBAC/MSCK at PPW2 and PPW4 and between PPW2 and PPW4 for BBAC/BSCK and MBAC/MSCK. BBAC and MBAC were analysed by Wilcoxon Signed Ranks test to compare PPW2 with PPW4. However, Kruskal-Wallis test was used for the analysis between breed groups. BBAC and MBAC between each breed group were compared with Mann-Whitney-U Test. The ADMY was analysed using Mann-Whitney-U test between SCK positive and negative cows, including subgroups. Repeated measure ANOVA and Friedman tests were initiated for the analysis of milk yield between calving and postpartum 12 weeks.

Results

BBAC, MBAC, BCS and Parity in Different Dairies

The averages of BCS, BBAC and MBAC in the study cows were presented in *Table 1*. All cows lost BCS at PP30 and PP60 compared to calving BCS, except for PRP Simmental. BCS1 was not observed at calving. BCS2, BCS3 and BCS4 were detected in Holstein by 47%, 41%, and 8.6% at calving respectively. The average parity of Holstein, Montbeliard, Simmental and HC was 2.93±0.11 (n=37 PRP, n=179 MUL), 3.09±0.31 (n=5 PRP, n=18 MUL), 2.26±1.03 (n=9 PRP, n=29 MUL), 2.04±1.15 (n=11 PRP, n=12 MUL) respectively. The average parity of Simmental and HC was significantly lower (P<0.01) than Montbeliard and Holstein. *Fig. 1* and *Fig. 2* present BBAC and MBAC for different BCSs at calving. Calving-BCS determined significantly BBAC and MBAC at PPW2 and PPW4. Holstein having significantly high BBAC at PPW2 had BCS4 at PP30 and PP60. Significantly high BBAC at PPW2 was observed in Simmental having BCS4 at PP30 (P<0.01). Correlation coefficients (data not shown in

Table 1. *Beta-hydroxybutyric acid concentrations (BAC, x±se) in the blood (BBAC mmol/L) and milk (MBAC µmol/L) at postpartum week 2 and 4 (PPW2, PPW4) and body condition scores (BCS, x±se) at calving, postpartum day 30 (PP30) and 60 (PP60), in PRP and MUL Holstein, Montbeliard, Simmental and Holstein Crossbreed (HC)**

Cows	Groups	All Breeds	Holstein	Montbeliard	Simmental	HC	P (1)
All parities	BCS-calving	3.42±0.03[1]	3.35±0.03[a,1]	3.52±0.04[b,1]	3.59±0.10[b,1]	3.61±0.08[b,1]	0.00
	BCS-PP30	2.96±0.02[2]	2.94±0.03[a,2]	2.80±0.04[a,2]	3.24±0.08[b,2]	2.83±0.09[a,2]	0.00
	BCS-PP60	2.96±0.03[2]	2.88±0.03[a,3]	2.88±0.02[a,2]	3.41±0.09[b,1]	2.87±0.06[a,2]	0.00
	P(2)	0.00	0.00	0.00	0.01	0.02	-
	PPW2-BBAC	0.48±0.04	0.54±0.05[a]	0.32±0.07[b]	0.45±0.01[a]	0.03±0.04[c]	0.00
	PPW4-BBAC	0.42±0.03	0.46±0.04[a]	0.33±0.03[b]	0.44±0.04[a]	0.02±0.09[c]	0.00
	P(3)	0.21	0.11	0.43	0.95	0.48	-
	PPW2-MBAC	62.98±9.01	76.73±14.84[a]	52.17±9.68[a]	50.00±0.00[a]	-	0.45
	PPW4-MBAC	55.63±9.72	67.33±13.41[a]	8.70±4.04[b]	50.00±0.00[a]	-	0.00
	P(3)	0.00	0.03	0.00	1.00	-	-
PRP	BCS-calving	3.51±0.05[1]	3.48±0.06[a,1]	3.60±0.10[a,1]	3.56±0.19[a,1]	3.50±0.12[a,1]	0.76
	BCS-PP30	2.98±0.05[2]	2.98±0.05[a,2]	2.87±0.12[a,2]	3.17±0.17[a,1]	2.86±0.14[a,2]	0.44
	BCS-PP60	2.97±0.06[2]	2.92±0.07[a,2]	2.90±0.05[a,2]	3.33±0.17[b,1]	2.86±0.10[a,2]	0.03
	P(2)	0.00	0.00	0.01	0.08	0.00	-
	PPW2-BBAC	0.57±0.07	0.75±0.17[a]	0.52±0.30[b]	0.50±0.06[b]	0.03±0.02[c]	0.00
	PPW4-BBAC	0.40±0.07	0.51±0.12[a]	0.44±0.10[a]	0.42±0.08[a]	0.01±0.03[b]	0.00
	P(3)	0.04	0.08	1.00	0.20	0.16	-
	PPW2-MBAC	91.67±28.85	101.85±39.63[a]	70.00±20.00[a]	50.00±0.00[a]	-	0.57
	PPW4-MBAC	43.33±16.56	50.00±23.40[a]	10.00±10.00[a]	50.00±0.00[a]	-	0.10
	P(3)	0.00	0.03	0.06	1.00	-	-
MUL	BCS-calving	3.39±0.03[1]	3.32±0.03[a,1]	3.50±0.04[a,1]	3.60±0.12[b,1]	3.71±0.11[b,1]	0.00
	BCS-PP30	2.95±0.03[2]	2.93±0.03[a,2]	2.78±0.05[b,2]	3.27±0.10[c,2]	2.79±0.11[b,2]	0.00
	BCS-PP60	2.95±0.03[2]	2.87±0.04[a,3]	2.88±0.02[a,2]	3.45±0.11[b,2]	2.88±0.07[a,2]	0.00
	P(2)	0.00	0.00	0.00	0.00	0.00	-
	PPW2-BBAC	0.45±0.04	0.50±0.04[a]	0.27±0.03[b]	0.43±0.05[a]	0.03±0.03[c]	0.00
	PPW4-BBAC	0.42±0.03	0.45±0.04[a]	0.29±0.03[b]	0.45-0.04[a]	0.03±0.05[c]	0.00
	P(3)	0.68	0.36	0.41	0.57	1.00	-
	PPW2-MBAC	55.86±8.44	58.04±10.79[a]	47.22±11.05[a]	50.00±0.00[a]	-	0.56
	PPW4-MBAC	58.93±11.51	71.88±15.81[a]	8.33±4.52[b]	50.00±0.00[a]	-	0.00
	P(3)	0.01	0.21	0.01	1.00	-	-

PRP: *primiparous cows;* ***MUL:*** *multiparous cows; * MBAC was not tested in Holstein-Crossbreed (Holstein/Montbeliard);* ***P(1):*** *Kruskal-Wallis Test between breeds;* [a,b,c] *different letters refer significant difference (P≤0.05) in the same line between breeds;* ***P(2):*** *Kruskal-Wallis Test between BCS check times (calving, PP30, PP60);* [1,2,3] *different numbers refers significant difference (P≤0.05) in the same column between BCS-calving, BCS-PP30 and BCS-PP60;* ***P(3):*** *Wilcoxon Sig. Ranks test between PPW2 and PPW4*

tables) between BBAC and MBAC were r=0.60 and r=0.86 (P<0.05) at PPW2 and it was r=0.36 and r=0.14 (P>0.05) at PPW4 in Holstein and Montbeliard cows respectively. Correlation coefficients for BBAC and MBAC were r=0.45 and r=0.75 (P<0.05) between PPW2 and PPW4 in Holstein respectively. No significant correlation was found in other breeds.

Prevalence of BSCK and MSCK in Holstein

SCK prevalence analysis was not performed in the breeds other than Holstein, none of Simmental and HC cows have exceed the cut-point of BHBA for SCK definition. Out of 23 Montbeliard cows, 1 PRP Montbeliard with BCS4 at calving showed BSCK at PPW2 only, but that cow became negative at PPW4. BSCK was detected in Holstein farms (farms 2, 3, 5, 6, 7 and 8) at PPW2 and 4 respectively. The difference between PPW2 and PPW4 was significant (P<0.01). Holstein farms 1 and 4 were negative for BSCK. The descriptive data about the prevalence of BSCK and MSCK, their relation with the parity and BCSs were presented in *Table 2* for Holstein. PRP Holstein with BSCK at PPW2 and 4, lost significantly (P<0.05) BCS at PP60. The difference between PPW2 and PPW4 was significant (P<0.01) in MSCK1/2 and MSCK2 prevalence. MSCK2 prevalence was negative in Holstein farms 1, 3 and 4. No significant difference was found in BCSs of MSCK2

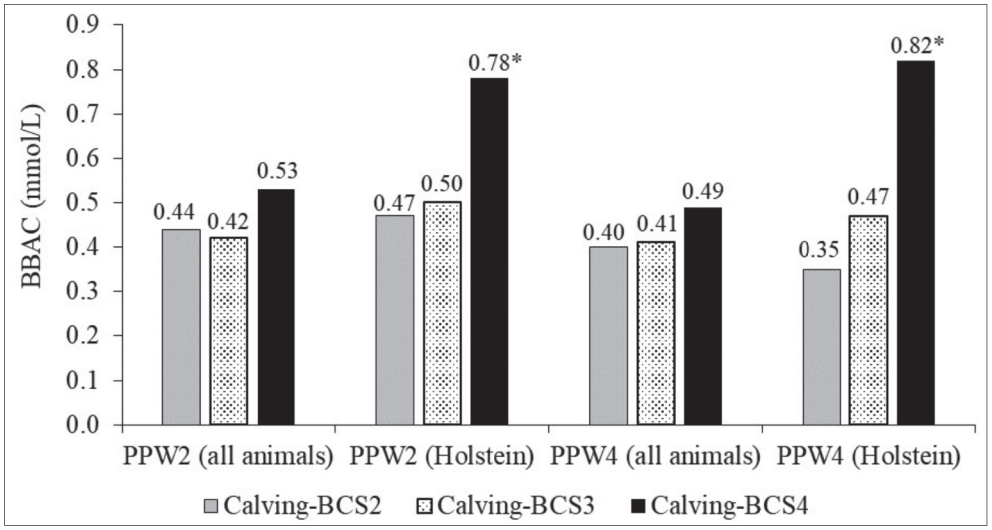
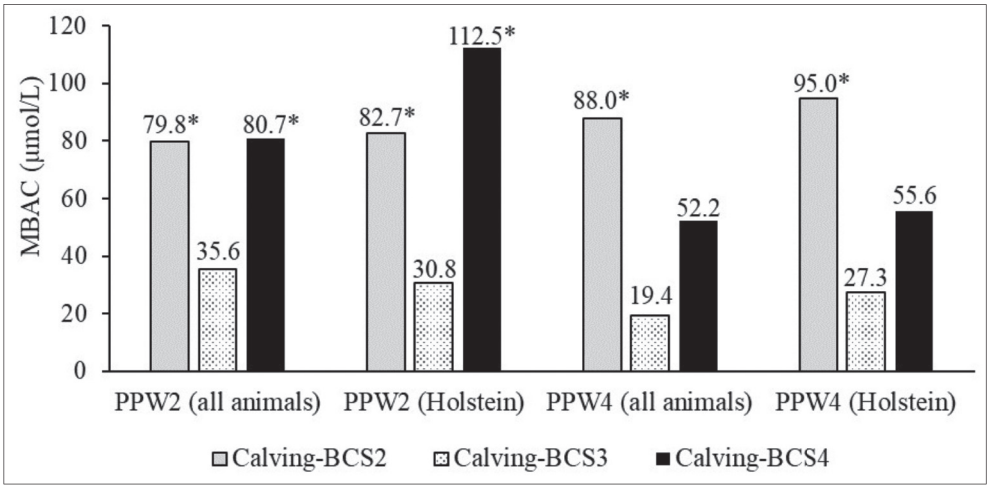


Fig 1. Average blood beta-hydroxy-butyric acid concentrations (BBAC) at postpartum week 2 (PPW2) and postpartum week 4 (PPW4) in all study cows and separately in Holstein that had different body conditions scores (BCS) at calving. * P≤0.05 compared to BCS2 and BCS3 at the respective week

Fig 2. Average milk beta-hydroxy-butyric acid concentrations (MBAC) at postpartum week 2 (PPW2) and postpartum week 4 (PPW4) in all study cows and separately in Holstein that had different body conditions scores (BCS) at calving. * P<0.01, significantly different from BCS3



positive MUL Holstein cows between calving, PP30 and PP60. However, all PRP cows with positive MSCK lost BCS between calving, PP30 and PP60. The combined prevalence of BSCK/MSCK1 and BSCK/MSCK2 was 4.0 and 2.0% at PPW4 in Holstein cows respectively. The prevalence of BSCK/MSCK1/2 was 8.6 and 4.0% at PPW2 and PPW4 in Holstein respectively. The difference between PPW2 and 4 was significant (P<0.01). The percentage of BSCK, MSCK1 and MSCK2 positive cases at both PPW2 and PPW4 were 2.3, 5.9 and 3.0% in Holstein respectively. No correlation for BSCK and MSCK was found between PPW2 and 4 (P>0.05). The correlation coefficient was r=0.34 (P>0.05) for BSCK between PPW2 and PPW4 in Holstein. Correlation coefficients were r=0.26 and r=0.48 (P>0.05) for MSCK1 and MSCK2 between PPW2 and PPW4 in Holstein respectively.

Postpartum Health Disorders and Culling in Holstein

The incidence of PPHD except culling rate and its association with SCK were presented in Holstein in *Table 3*. MSCK1 did not significantly correlate with PPHD in Holstein and therefore it was not presented. The overall culling rate was 3.7% among Holstein in 90DIM. Holstein that were positive for MSCK1, MSCK1/2 and MSCK2 at PPW2 created a likelihood of 6.3, 12.5 and 25% for culling risk respectively. MSCK2 positive cows were significantly more likely (odds ratio: 11.20, P<0.05) to be culled than even MSCK1/2 (odds ratio: 3.46). MSCK1 did not create a significant risk for culling. The average BCS of culled Holstein was normal (BCS3) at calving, but a significantly BCS loss was observed at PP30 (BCS1). The difference between BCSs of culled Holstein (2.48±0.20) and non-culled Holstein (2.95±0.03) was significant at PP30 (P=0.026). The average parity of culled Holstein was 3.62 (one PRP, 7 MUL). Mastitis (n=1), metritis (n=1) and displaced abomasum (n=1), multiple diseases (n=1, 12%), lameness (n=2, 25%) were observed in culled Holstein. Clinical ketosis, displaced abomasum, metritis, mastitis, lameness and multiple diseases were observed moderately and, in some cases, significantly higher in Holstein that were positive for BSCK, MSCK1/2 and MSCK2 at PPW2 or 4 *(Table 3)*. Displaced abomasum and cystic ovarian incidence did not correlate significantly with SCK in Holstein. BSCK and MSCK2 positive Holstein at PPW2

Table 2. *Descriptive statistic about the prevalence of subclinical ketosis (SCK) at PPW2 and PPW4 in PRP and MUL Holstein and their body condition scores (BCS, mean, minimum and maximum) at calving, postpartum day 30 (PP30) and 60 (PP60)*

Total Tested			Parity		BCS (mean, min and max)		
SCK Group	**n**	**SCK (%)**	**PRP/MUL**	**%**	**Calving**	**PP30**	**PP60**
BSCK-PPW2	216	8.3	PRP	27.7	3.44 (3.00-4.00)	3.10 (3.00-3.50)	2.95 (2.50-3.50)
			MUL	72.2	3.33 (2.50-4.00)	3.16 (2.50-4.50)	3.32 (2.50-5.00)
BSCK-PPW4	213	4.7	PRP	30.0	3.58 (3.00-4.00)	3.25 (3.00-3.50)	3.00 (2.50-3.50)
			MUL	70.0	3.70 (3.50-4.00)	3.43 (3.00-5.00)	3.50 (3.00-5.00)
MSCK1-PPW2	139	11.5	PRP	12.5	3.50 (3.50-3.50)	2.85 (2.70-3.00)	2.77 (2.75-2.80)
			MUL	87.5	3.35 (2.50-4.00)	2.91 (2.50-3.50)	2.87 (2.50-3.50)
MSCK1-PPW4	101	4.9	PRP	20.0	3.75 (3.75-3.75)	3.25 (3.25-3.25)	3.50 (3.50-3.50)
			MUL	80.0	3.20 (3.00-4.00)	2.88 (2.50-3.50)	2.67 (2.50-3.00)
MSCK2-PPW2	139	5.8	PRP	50.0	3.60 (3.00-4.00)	3.16 (3.00-3.50)	3.16 (3.00-3.50)
			MUL	50.0	3.25 (3.20-3.50)	3.25 (2.50-4.00)	3.40 (2.75-4.00)
MSCK2-PPW4	101	6.9	PRP	14.3	3.20 (3.20-3.20)	3.00 (3.00-3.00)	3.00 (3.00-3.00)
			MUL	85.7	3.30 (3.20-3.50)	3.36 (3.00-5.00)	3.28 (2.75-5.00)
MSCK1/2-PPW2	139	17.3	PRP	25.0	3.58 (3.50-4.00)	3.00 (2.60-3.50)	3.00 (2.75-3.50)
			MUL	75.0	3.33 (2.50-3.50)	2.98 (2.50-4.50)	2.98 (2.50-4.50)
MSCK1/2-PPW4	101	11.9	PRP	16.7	3.50 (3.20-3.75)	3.12 (3.00-3.25)	2.75 (2.50-3.00)
			MUL	83.3	3.25 (2.50-4.00)	3.18 (2.50-5.00)	3.10 (2.50-5.00)

BSCK: *blood beta-hydroxybutyric acid concentration ≥1.20 mmol/L;* ***MSCK1:*** *milk beta-hydroxybutyric acid concentration = 100 µmol/L;* ***MSCK2:*** *milk beta-hydroxybutyric acid concentration ≥200 µmol/L;* ***MSCK1/2:*** *milk beta-hydroxybutyric acid concentration ≥100 µmol/L;* ***PRP:*** *primiparous;* ***MUL:*** *multiparous;* ***PPW2:*** *postpartum week 2;* ***PPW4:*** *postpartum week 4*

Table 3. *Incidences of postpartum health disorders (PPHD) in Holstein cows that were tested positive or negative for subclinical ketosis (SCK)*

SCK group	SCK		PPHD (%)								
	Positive/ Negative	**%**	**CK**	**RP**	**DA**	**Met**	**Mast**	**Lam**	**MF**	**CO**	**MD**
BSCK-PPW2	negative	91.7	0.0	2.5	1.5	6.6	17.7	11.1	1.0	3.5	6.6
	positive	8.3	44.2[1]	0.0	0.0	5.6	11.1	22.2	0.0	0.0	5.6
BSCK-PPW4	negative	95.3	1.5	2.5	1.0	6.4	17.2	10.3	1.0	3.4	6.4
	positive	4.7	20.0[2]	0.0	10.0[4]	10	20.0	30.0[6]	0.0	0.0	10.0
MSCK1/2-PPW2	negative	82.7	0.9	2.6	1.7	7.0	11.3	7.8	1.7	4.3	8.7
	positive	17.3	16.7	0.0	0.0	12.5	12.5	16.7	0.0	0.0	8.3
MSCK1/2-PPW4	negative	88.1	2.2	0.0	1.1	7.9	10.1	5.6	1.1	5.6	7.9
	positive	11.9	8.3	0.0	8.3	25.0[5]	33.3[1]	16.7	0.0	0.0	25.0[7]
MSCK2-PPW2	negative	94.2	1.5	2.3	1.5	7.6	10.7	9.9	1.5	3.8	0.8
	positive	5.8	37.5[3]	0.0	0.0	12.5	25.0	0.0	0.0	0	12.5
MSCK2-PPW4	negative	93.1	2.1	0.0	2.1	9.6	11.7	7.4	1.1	5.3	9.6
	positive	6.9	14.3	0.0	0.0	14.3	28.6	0.0	0.0	0.0	14.3

[1] P<0.05; [2,3] P<0.01; [4] P=0.09; [5,6,7] P=0.06; ***PPW2:*** *postpartum week 2;* ***PPW4:*** *postpartum week 4;* ***BSCK:*** *blood beta-hydroxybutyric acid concentration (BAC) ≥1.20 mmol/L;* ***MSCK2:*** *milk BAC ≥200 µmol/L;* ***MSCK1/2:*** *milk BAC ≥100 µmol/L;* ***CK:*** *clinical ketosis;* ***RP:*** *retained placenta;* ***DA:*** *displaced abomasum;* ***Met:*** *metritis;* ***Mast:*** *mastitis;* ***Lam:*** *lameness;* ***MF:*** *milk fever;* ***CO:*** *cystic ovarian;* ***MD:*** *multiple diseases. No significant relation was found between MSCK1 and PPDH*

or 4 were more likely to developing clinical ketosis (odds ratio: 15.4, P<0.05). Holstein with positive SCK had frequently metritis, however, the incidence was most remarkable in MSCK1/2 positive Holstein at PPW4 (P=0.06, odds ratio: 4.48). Those cows had also a moderate significantly (P=0.07) lower BCS (2.81) at PP60 than other cows. Holstein with MSCK1/2 at PPW4, were more likely to have mastitis (odds ratio: 5.08, P<0.05) *(Table 3)*. Holstein with BSCK at PPW4 had a moderate significantly high incidence of lameness (odd ratio: 4.25, P=0.06).

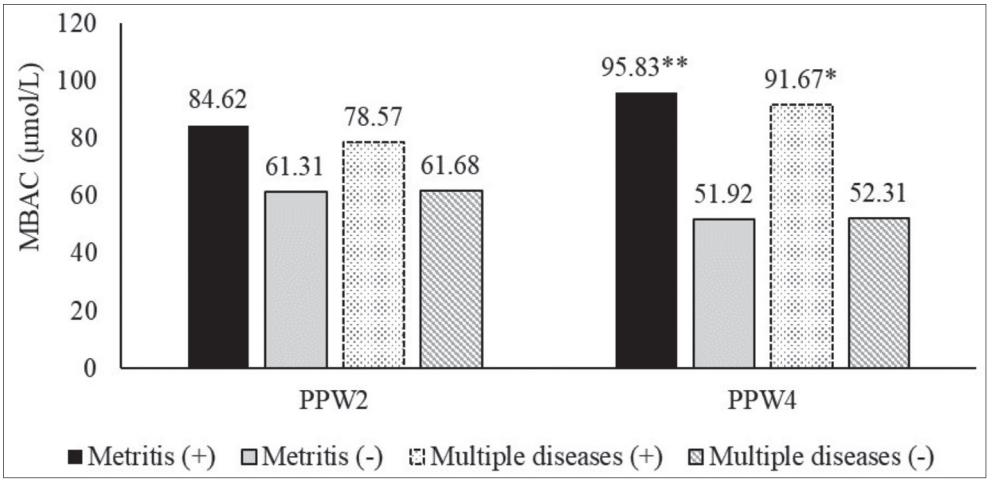


Fig 3. Average milk beta-hydroxybutyric acid concentrations (MBAC) at postpartum week 2 (PPW2) and postpartum week 4 (PPW4) in cows having metritis and multiple diseases throughout the study period. Based on Mann-Whitney Test; * P≤0.05 versus multiple disease negative, ** P<0.01 versus metritis negative

Table 4. *Average daily milk yield of Holstein (x±se, kg) with positive and negative subclinical ketosis (SCK) at postpartum week 2 or 4 (PPW2 or PPW4) in PRP and MUL Holstein cows in 90DIM*

SCK Group	All Holstein			PRP			MUL		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
BSCK at PPW2	34.62±1.55	38.16±0.65	0.10	33.55±2.79	40.59±1.45	0.05	35.16±1.92	37.70±0.71	0.33
n	18	188		6	30		12	158	
BSCK at PPW4	35.05±2.19	37.92±0.63	0.43	33.60±3.23	39.95±1.42	NA	36.24±1.80	37.56±0.72	0.68
n	11	192		3	33		8	159	
MSCK2 at PPW2	32.46±2.48	38.02±0.75	0.07	29.71±0.66	37.03±1.21	NA	34.11±3.91	38.25±0.88	0.31
n	8	126		3	23		5	103	
MSCK2 at PPW4	33.85±11.87	36.70±10.88	0.47	28.39	36.63±1.29	NA	35.21±1.66	36.72±1.07	NA
n	5	97		1	21		4	76	

PRP: *primiparous;* ***MUL:*** *multiparous;* ***90DIM:*** *90 days in milk;* ***NA:*** *not applicable due to low number of cases;* ***BSCK:*** *blood beta-hydroxybutyric acid concentration ≥1.20 mmol/L;* ***MSCK2:*** *milk beta-hydroxybutyric acid concentration ≥200 µmol/L*

MSCK1/2 positive Holstein at PPW4 showed an incidence of 25% multiple diseases (odds ratio: 4.5, P=0.06) *(Table 3)*. Average MBAC at PPW4 were significantly higher in cows having metritis and multiple diseases *(Fig. 3)*. BBAC at PPW2 was significantly higher in cows with metritis (P<0.05) in 90DIM (data not shown in tables).

Association of BSCK and MSCK with Average Daily Milk Yield in Holstein

The comparison of ADMY between SCK positive and negative Holstein was presented in *Table 4, 5, 6* and *Fig. 4* and *5*. The average daily, weekly and monthly milk production of BSCK and MSCK2 negative Holstein had always an upwards trend throughout 12 weeks postpartum in comparison to positive cows *(Table 4, 5, 6* and *Fig. 4* and *5)*. The difference between SCK negative and positive cows regarding the ADMY was significant at weeks 10th, 11th and 12th *(Fig. 4* and *5)*. MSCK2 positive Holstein cows at PPW2 had moderate significantly (P=0.07) lower ADMY *(Table 4)*. The ADMY was significantly different in Holstein that was in combined prevalence groups of BSCK and MSCK2, and roughly 5.4 kg and 4 kg higher ADMY was observed in SCK negative cows than positive cows respectively *(Table 5)*. If Holstein cows were positive both for BSCK and MSCK2 at PPW2, ADMY was 6.7 kg less than negative cows, which was significant (P≤0.05) *(Table 5)*. MSCK1 and MSCK1/2 in Holstein did not have a significant effect on average daily, weekly and monthly milk yield, even no effect was observed in the combined prevalence groups. Therefore, the data was not presented in the tables. The ADMY of PRP Holstein without BSCK at PPW2 was significantly higher (P≤0.05) than those with BSCK, which meant an average 7 kg milk yield loos per day *(Table 4)*. Holstein with a negative BSCK and MSCK2 at PPW2 had a much higher ADMY in the second and third months postpartum *(Table 6)*. BSCK negative PRP Holstein at PPW2 had 13.6 kg more ADMY in the second month of postpartum that was significantly different (P≤0.05) from the positive cows (data not shown in tables).

Discussion

The present study reported a lower prevalence of BSCK in Holstein than the previous studies by other reports in European countries [11], Turkey [11,12] and worldwide [13], although the same cut-off point of BBAC for BSCK was

Table 5. *Average daily milk production of Holstein (x±se, kg) with positive and negative subclinical ketosis (SCK) in the combined groups at postpartum week 2 or 4 (PPW2 or PPW4) in 90DIM*

SCK Group	Milk Yield		P	SCK (%)	Not Matching to Group* (n/%)
	SCK Positive	SCK Negative			
BSCK at PPW2 or 4	34.25±1.44	38.24±0.65	0.05	11.6	0
n (206)	24	182			
MSCK2 at PPW2 or 4	32.68±12.02	38.10±10.76	0.05	7.6	0
n (tot:134)	10	124			
BSCK/MSCK2 at PPW2	31.33±3.23	37.99±0.79	0.05	4.5	9/6.7
n (tot: 134)	6	119			
BSCK/MSCK2 at PPW4	36.7	36.56±0.85	NA	0.98	8/7.8
n (tot: 102)	1	93			
BSCK or MSCK2 at PPW2	34.74±1.39	38.19±0.65	0.09	8.9	0
n (tot: 134)	12	122			
BSCK or MSCK2 at PPW4	34.88±1.26	38.07±0.65	0.17	8.8	0
n (tot: 102)	9	93			

90DIM: *90 days in milk;* ***NA:*** *not applicable;* ***BSCK:*** *blood beta-hydroxybutyric acid concentration ≥1.20 mmol/L;* ***MSCK2:*** *milk beta-hydroxybutyric acid concentration ≥200 µmol/L; * These animals cannot be allocated in the respective group because they were positive for one of SCK only*

Table 6. *Average daily milk production (x±se) (kg) per month of all Holstein tested for positive (+) or negative (-) of subclinical ketosis (SCK) in the blood (BSCK) and milk (MSCK2) at postpartum week 2 or 4 (PPW2 or 4)*

SCK Group	SCK	n	First Month	Second Month	Third Month	P(1)
BSCK at PPW2	+	18	32.77±1.50	34.58±2.54	35.08±2.35	0.03
	-	188	34.74±0.54	39.86±0.76*	39.89±0.77**	0.00
MSCK2 at PPW2	+	8	30.25±2.36	30.74±5.12	32.92±4.27	0.16
	-	126	34.42±0.67	39.91±0.89*	39.74±0.85**	0.00
BSCK at PPW4	+	11	33.98±1.43	34.31±3.86	36.01±1.63	0.27
	-	192	34.58±0.54	39.65±0.75	39.67±0.78	0.00
MSCK2 at PPW4	+	5	28.22±2.14	36.51±2.67	36.81±2.11	0.07
	-	97	33.61±0.73	38.38±1.04	38.11±1.05	0.00

P(1): *difference between average milk productions of month 1, 2, 3; average milk yield of the first month is significantly lower than month 2nd and 3rd where it is applicable (P value); * P≤0.05; ** P=0.07 between SCK positive and negative groups at the respective testing month;* ***BSCK:*** *blood beta-hydroxybutyric acid concentration ≥1.20 mmol/L;* ***MSCK2:*** *milk beta-hydroxybutyric acid concentration ≥200 µmol/L*

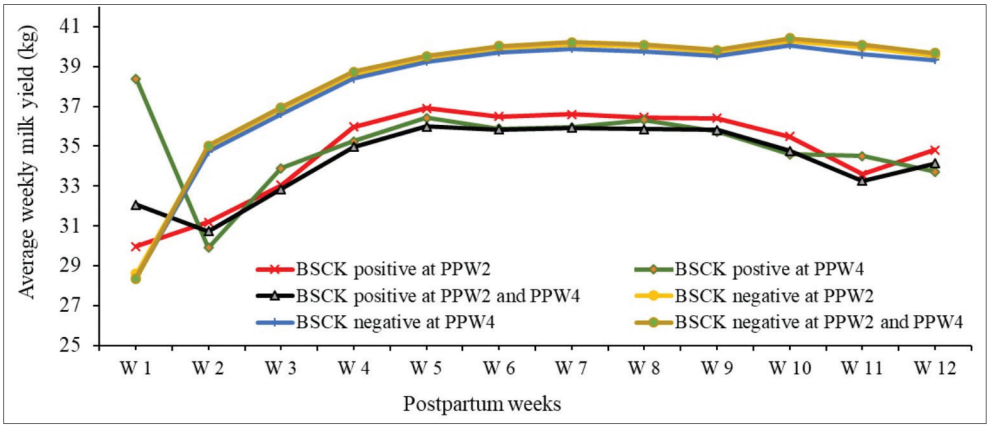


Fig 4. Average daily milk yield per week of Holstein with positive or negative subclinical ketosis (SCK) at postpartum week 2 or 4 (PPW2 or 4) in the blood (BSCK: blood beta-hydroxybutyric acid concentration ≥1.2 mmol/L). Remark: line of BSCK negative cows at 'PPW2 and PPW4' overlaps the line of BSCK negative cows at PPW2

used in all these studies. Even, BSCK prevalence was little higher using the lower cut-point of BBAC (≥1.0 mmol/L) in Turkey [27,28]. Others defined BSCK by a threshold level of BBAC ≥0.96 mmol/L [29], ≥1.0 mmol/L [5,30] and ≥1.4 mmol/L [15,19,24]. But, the cut-point of BBAC≥1.20 mmol/L for BSCK definition was found the most acceptance

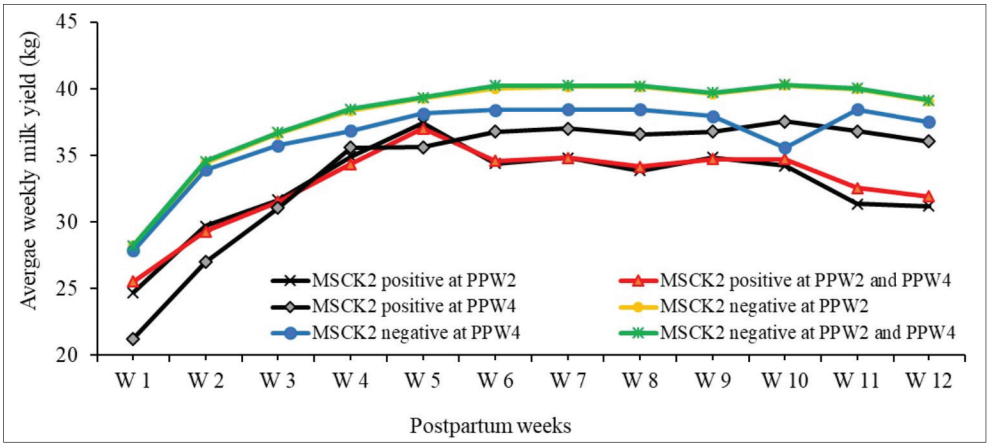


Fig 5. Average daily milk yield per week of Holstein with positive or negative subclinical ketosis (SCK) at postpartum week 2 or 4 (PPW2 or 4) in the milk (MSCK2: milk beta-hydroxybutyric acid concentration ≥200 µmol/L). Remarks: The line of MSCK2 negative cows at 'PPW2 and PPW4' overlaps the line of MSCK2 negative cows at PPW2. The line of MSCK2 positive cows at 'PPW2 and PPW4' overlaps the line of MSCK2 positive cows at PPW2

which was much related to PPHD and milk production loss [3,7-9,11,13]. Therefore, the present study used also this cut-point of BBAC. The reason for this discrepancy in the results can be that no BSCK was detected in two Holstein farms, or a seasonal difference might be that a large part of the present study was conducted in the wintertime. Thus, this might indicate that there is no NEB developing in postpartum cows in these farms which resulted in a lower prevalence. Overall, significantly higher BSCK prevalence in Holstein and BBAC and MBAC in PRP cows were detected at PPW2 compared to PPW4 in the present study. This was in compromise with other studies, which reported that the first two weeks postpartum are the most prevalent and critical time points for SCK in Holstein cows [11,13,22-24]. Most BSCK positive cows were MUL cows. This was consistent with other studies [13,31-33]. However, there are also not consistent reports in Holstein [24,34] and in Jersey [35]. The peak prevalence of SCK occurred in the third and fourth week of lactation [4], this result was slightly close to the present study. Similarly, Carrier et al.[24] reported a higher prevalence at PPW1 and 2 than PPW3 and thereafter. It seems to be that BBAC testing should be done earlier but not later than PPW2. Therefore, the test points of the present study set for BAC analysis at PPW2 and PPW4 in the blood and milk was consistent with most of the previously reported studies. The present study reported differently from others an individual BBAC/BSCK and MBAC/MSCK at PPW2 and PPW4 exactly, while many of studies reported at not an exact time point postpartum [11,13,19,28,32,36]. Fat Holstein at calving had significantly higher BBAC at PPW2 and 4 and MBAC at PPW2 in the present study. This was in compromise with the previous reports [33,37]. Differently, the present study showed that all thin cows and thin Holstein at calving had higher MBAC at PPW4. The majority of cows lost BCS in PP30 and 60 compared to calving BCS. PRP Holstein with positive BSCK or MSCK lost BCS within 60 days postpartum. Probably, these cows had fat mobilisation due to NEB to compensate energy requirement for the first milk production [19]. The prevalence rate of MSCK in the present study was different due to different cut-points of MBAC. The cut-off value of MBAC for the definition of MSCK in the milk varied among the studies [9,14-16,18]. MBAC≥200 µmol/L was recommended to define cows having severe and positive ketosis [9] and used also in the previous studies [15,18]. Both MSCK1 and MSCK2 prevalence rate was low compared to the literature [14-16] although it was tested at two different postpartum time points. But, MBAC of MUL cows increased significantly at PPW4 compared to PPW2. Consequently, MSCK2 positive Holstein were more likely to develop clinical ketosis although other PPHD such as metritis, mastitis and multiple diseases were observed. SCK was frequently associated with PPHD in Holstein [3,7,8,11,13]. The present study showed that BSCK at PPW4 caused a risk for displaced abomasum and lameness without a high significant odd. BSCK positive Holstein at PPW2 and 4 were more likely to develop clinical ketosis. Transformation of SCK into clinical ketosis was frequently reported at a high risk factor and likelihood [2,19,37]. BSCK positive Holstein at PPW4 had a nonsignificant incidence of displaced abomasum, because displaced abomasum was overall low in study cows. Displaced abomasum was the most frequently detected metabolic disorder related to SCK in the first weeks after calving with a high-risk factor [11,19,37]. Mastitis incidence was frequently high in SCK positive Holstein in the present study. This finding was in line with others [6,8,13], but Suthar et al.[11] did not observe significant risk for mastitis. Differently from others, cows with mastitis have got significantly lower BCS (thin) at calving. Kremer et al.[38] stated that cows with BBAC >1.4 mmol/L were more susceptible to severe mastitis in an experimental *E. coli* study. MSCK1/2 at PPW4 created a high risk for metritis in Holstein and those cows had significantly high MBAC at PPW4. Similarly, a high metritis incidence was already reported in Turkey and worldwide [2,6,11,13,19]. The retained placenta and milk fever as well as cystic ovarian were not observed in Holstein with SCK in the present study. A cut-point of BBAC for this early PPHD wasn't established previously [11]. Others reported high risks for retained

placenta in association with BSCK [13,37]. The cystic ovarian associated with BSCK was reported by previous reports [4,31]. Lameness incidence was high and created moderately significant risk in BSCK positive Holstein at PPW4. Similar findings were observed by others in BSCK positive Holstein [11,13]. Other study [6] did not report lameness in SCK or clinical ketosis positive Holstein in Turkey. MSCK1/2 and MSCK2 positive cows at PPW4 were significantly more likely to be culled and having metritis and multiple diseases. Similar findings were reported in BSCK and in clinical ketosis in Turkey previously [6]. The present study found culling rates in MSCK positive cows and a positive correlation between multiple diseases, metritis and high MBAC additionally. The present study indicated a reduced ADMY in BSCK and MSCK2 positive Holstein which was consistent with the finding of previously reported studies [4,8,19,32,36]. MSCK2 positive Holstein had 6.7 kg ADMY loss in 90DIM, it was 4 kg for cows with positive BSCK at PPW2 or 4. Increasing BBAC above 1.0 mmol/L during PPW2 was associated with progressively less milk yield [19]. A linear negative effect of BBAC beginning at 1.2 mmol/L at PPW1 was observed on milk production [19]. This can result in economic losses throughout the production cycle [7,8,17], e.g., in an average of USD 200-290 per cow [3]. Differently from other reports, BSCK at PPW2 resulted in significantly lower ADMY in PRP Holstein. Similar trend was observed in MSCK2 positive PRP Holstein, but the number of animals was not enough to do comparison. Probably, these PRP cows suffered a poor adaptive response to the onset of the first lactation and the resulting NEB [19]. A little difference was observed between PRP and MUL cows in hyperketonemia incidence [34]. Chandler et al.[35] found more prevalent hyperketonemia in PRP than MUL Jersey cows. A very small difference in ketosis prevalence between PRP and MUL cows was found in Ayrshire and Friesian cows [39]. This might lead to the point that PRP Holstein needs more intense care in early lactation to overcome NEB and adapt to the first lactation. However, this was not observed at ADMY between MSCK1 positive and negative Holstein. The lower threshold level of MBAC (≥100 µmol/L) for MSCK1 and MSCK1/2 definition at PPW2 or PPW4 did not significantly affect ADMY in Holstein. Previous studies [4,36] reported the association between MSCK1 and reduced milk production in Holstein. A positive moderate correlation between MBAC and BBAC was observed in Holstein, but not in other breeds. Especially, the correlation was high in terms of changes of BBAC and MBAC between PPW2 and PPW4. This correlation between BBAC and MBAC was observed by others [15]. In contrast, this correlation was not confirmed between BSCK and MSCK neither at PPW2 and PPW4. The reason might be due to the lower sensitivity of milk BHBA test strips compared to cow-side blood BHBA analysers [24]. Semiquantitative determination of MBAC which based on the colour indication for BAC might affect the results. It was stated that concentrations of milk and blood BAC were poorly correlated and the use of milk strips overestimated the concentrations of BAC in the milk [40]. The lack of relationship between MBAC and BBAC was observed by Andersson [41] and they suggested that milk BAC could be of low value for the detection of SCK, so that few authors presented a critical cut-off point for MBAC. BHBA can be utilised by the mammary gland for fatty acids synthesis and converted to butyrate [22,42] that is why MBAC is only 10% to 15% of BBAC, possibly because of the ketone body's role in fat metabolism in the udder [41]. These fluctuations in MBAC in contrast to BBAC may be a reason for the difference of BSCK from MSCK1 and MSCK2 in the present study. But, the prevalence of MSCK in Holstein was often reported higher than the prevalence of BSCK [9,14]. Nevertheless, the specificity and sensitivity of the milk test strips used in the present study were confirmed for MBAC in cows [24], but there were still possibilities to observe around 3-5% false positive and false negative cases, which need to be taken into account by interpreting the milk results. That was the reason why both blood and milk tests were performed for the detection of BSCK and MSCK in the present study. Low BBAC and MBAC in Montbeliard and HC compared to Holstein and Simmental was an important outcome of this study. SCK was not found in Simmental and HC cows. One Montbeliard exceeded the cut-point of BBAC at PPW2 but not at PPW4. That was irrelevant because no prevalence analysis was conducted due to the small sample number in these breeds and that was also not aimed. In contrast to the present study, a study [43] reported a high incidence of BSCK in Simmental that were in early lactation and late pregnancy. A significantly reduced milk yield was observed in Montbeliard with BSCK in the second month of lactation [44]. Simmental cows were mostly culled because of sterility and reproductive diseases, but Montbeliard cows were culled due to poor yield and udder problems [45]. The reason for the discrepancy between the results of the studies might be management system differences and parity effect. Thus, the average parity was quite low for Holstein-Crossbreed and Simmental in the present study. Gantner et al.[46] found that the highest prevalence risks of ketosis were observed in 20 DIM of PRP Simmental, parity 2 and 3 cows. The French Simmental family has three strains; Pie rouge de l'Est (or French Simmental), Montbeliard and Abondance [20]. Thus, Montbeliard and Simmental cows were classified in the same family of French Simmental [20,21] and they might show a certain extent resistance to SCK [46], especially it can be much obvious under modern management system. Strong resistance of this dual-purpose Simmental Flechvieh breeds to mastitis was reported [20]. Although the number of Flechvieh cows looked small compared to Holstein for

a prevalence analysis in the present study, the resulting evidences about BBAC and MBAC might show the overall trend for SCK development associated to NEB in those breeds. Holstein lost BCS throughout the study period, although other breeds lost BCS at PP30 compared to calving only. Even, Simmental's average BCS at PP60 was similar to calving. This resulted for Holstein in higher BBAC at PPW2 and PPW4 than other breeds with exception of Simmental. No significant change was observed in BBAC between PPW2 and PPW4 in any breeds, however overall MBAC was much higher at PPW2 than PPW4 in PRP Holstein and Montbeliard. Taking all breeds parities into account, it can be speculated that MBAC showed the BCS losses better than BBAC. BCS losses are the result of fat mobilisation due to NEB [3].

In conclusion, BSCK and MSCK were still a herd problem causing PPHD, culling and ADMY loss in the Holstein farms in Turkey. The prevalence of SCK was much higher at PPW2 than PPW4 and fat cows at calving were more likely to have high BBAC and emaciated and fat cows showed much higher MBAC that was associated with metritis and multiple diseases. BSCK and MSCK2 positive Holstein at PPW2 had an ADMY loss of 6.7 kg. ADMY loss was 7 kg in PRP Holstein with BSCK at PPW2. The cut-off point of MBAC≥100 µmol/L did not cause a significant effect on ADMY. However, a higher cut-off point of MBAC≥200 µmol/L (MSCK2) caused a significantly reduced ADMY. BCS losses were better reflected with an increased MBAC at PPW2 in all related breeds. Simmental and related breeds (Flechvieh breeds) might have a certain resistance to SCK, therefore SCK prevalence and its effect on ADMY and PPHD need to be investigated in much larger samples' sizes in all related breeds. PRP Holstein needs to be investigated more intensively in terms of the development of NEB and associated ADMY performance under current modern conditions and high expectations for milk production.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author on a reasonable request.

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Competing Interests

Authors declare there are no conflicts of interest in the present study.

Author Contributions

Experimental design and data collection were conceived by KA, AD and ACO. Statistical analysis was conducted by SD and validated by ACO. Original draft was written by AD and KA. All authors have contributed to the revision and final proof-reading of the manuscript.

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RESEARCH ARTICLE

Serum and Intestinal Tissue Zonulin Levels in the Evaluation of Intestinal Permeability in Rats with Acute Pancreatitis

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Abstract: To evaluate the effects on malondialdehyde (MDA), superoxide dismutase (SOD), lipid hydroperoxide (LOOH), glutathione (GSH) levels as oxidative stress markers, zonulin levels, and histopathological findings of experimental acute pancreatitis (AP). This study was conducted three groups of Sprague-Dawley rats (seven animals in every group). First group was evaluated as control group (sham laparotomy). Group 2 (AP, 48% ethyl alcohol) and group 3 (severe pancreatitis (SP), 80% ethyl alcohol) was performed. The effects of pancreatitis were evaluated by comparing these groups according to histopathologial results of polimorphonuclear leukocytes, infiltration, oedema, haemorrhagie, apoptosis, aciner cellular degeneration in pancreatic and intestinal tissue. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected. MDA, LOOH and zonulin levels in serum and intestinal tissue were found to be significantly higher in the SP group compared to the control group. The serum and intestinal tissue SOD and GSH were found to be significantly lower than the control group. In the AP group, while LOOH and zonulin in tissues were significantly higher than control, only zonulin levels in serum were found higher than control. Zonulin and oxidative stress is basically involved in the pathogenesis of pancreatitis. Biochemical results are also supported by histopatological improvement in intestinal and pancreas tissue. Patients with pancreatitis may be more exposed to impaired gut barrier function. Serum zonulin levels can be used in the evaluation of intestinal permeability in AP.

Keywords: *Experimental acute pancreatitis, Intestinal permeability, Lipid hydroperoxide, Malondialdehyde, Zonulin*

Akut Pankreatitli Sıçanlarda Bağırsak Geçirgenliğinin Değerlendirilmesinde Serum ve Bağırsak Dokusu Zonulin Düzeyleri

Öz: Deneysel akut pankreatitin (AP) histopatolojik bulguları ile zonulin düzeyleri ve oksidatif stres belirteçleri olan malondialdehit (MDA), süperoksit dismutaz (SOD), lipid hidroperoksit (LOOH) ve glutatyon (GSH) düzeyleri arasındaki ilişkinin değerlendirilmesi amaçlanmıştır. Bu çalışmaya, üç grup olacak şekilde Sprague-Dawley sıçanı (her grupta yedi hayvan) alınmıştır. Birinci grup kontrol grubu (sham laparotomi) olarak belirlendi. Grup 2 (%48 etil alkol) AP ve grup 3 (%80 etil alkol) ağır (şiddetli) pankreatit (ŞP) olarak tasarlandı. Pankreatitin etkileri, pankreas ve bağırsak dokusunda bu gruplar karşılaştırılarak histopatolojik sonuçlara göre polimorfonükleer lökositler, infiltrasyon, ödem, hemoraji, apoptoz, asiner hücresel dejenerasyon değerlendirildi. ŞP grubunda tamamen düzleşmiş mukozal yüzey ve ciddi villus kaybı (total villus atrofisi), lamina propriada kriptlerde dezorganizasyon ve hiperplazi tespit edildi. Serum ve bağırsak dokusunda MDA, LOOH ve zonulin düzeyleri kontrol grubuna göre ŞP grubunda anlamlı olarak yüksekti. Serum ve bağırsak dokusu SOD ve GSH değerleri kontrol grubuna göre anlamlı düşük bulundu. AP grubunda dokulardaki LOOH ve zonulin düzeyleri kontrol grubuna göre anlamlı yüksek bulunurken, sadece serum zonulin düzeyleri kontrole göre daha yüksekti. Zonulin ve oksidatif stres esas olarak pankreatit patogenezinde rol oynar. Biyokimyasal sonuçlar ayrıca bağırsak ve pankreas dokusundaki histopatolojik iyileşme ile de desteklenmektedir. Pankreatitli hastalar bozulmuş bağırsak bariyer fonksiyonuna daha fazla maruz kalabilirler. AP'de bağırsak geçirgenliğinin değerlendirilmesinde serum zonulin düzeyleri kullanılabilir.

Anahtar sözcükler: *Bağırsak geçirgenliği, Deneysel akut pankreatit, Lipid hidroperoksit, Malondialdehit, Zonulin*

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Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with tissue damage and necrosis, its incidence continues to increase worldwide and is the leading cause of hospitalizations [1-3]. AP has a mild form involving only the pancreas and a severe form that results in extra pancreatic organ failure associated with systemic inflammatory response syndrome and even death [1]. In addition, it is known that recurrent AP may cause chronic pancreatitis and as a result, exocrine and endocrine insufficiency may develop [4,5]. However, today there are no therapeutic agents that can change the course of the disease [1]. At the same time, our knowledge about the etiology and pathogenesis of the disease is limited. One reason for the limited information about pathogenesis is that lack of access to the human pancreatic organ during illness [1,2]. Nevertheless, significant advances have been made in recent years in elucidating the pathophysiological mechanisms of acute pancreatitis. Studies conducted in this context indicate that intestinal permeability increases in acute pancreatitis, but it is not clear whether this is a mechanism that causes AP or a just a consequence of the disease [3,6-8].

Epithelial tight junctions prevent allergens, toxins, and pathogens from entering the interstitial tissue through the epithelium in various organ systems. In the gastrointestinal tract, disruption of tight junctions and loss of epithelial barrier function increases intestinal permeability to harmful factors that cause inflammation and mucosal damage [9]. It is emphasized that systemic inflammatory syndrome and bacterial translocation developing in AP may develop after intestinal permeability increase [8].

The protein, zonulin, has been identified as an important regulator of intestinal permeability. When bound to surface receptors, intracellular actin filaments polymerize, and this process causes opening of tight junctions and increased intestinal permeability [10]. Dysregulation of the zonulin pathway followed by "intestinal leakage" due to increased intestinal permeability has been associated with the pathogenesis of many gastrointestinal, autoimmune, inflammatory and neoplastic diseases [11].

Another factor that may cause impairment of intestinal permeability is free oxygen radicals, mostly form of reactive oxygen species (ROS). ROS modulate multiple signaling pathways and disrupt tight junctions and damage the epithelial and endothelial barrier [9]. In an animal study, it was reported that in the early stage of acute pancreatitis, free radicals derived from cytotoxic oxygen can contribute to the improvement of changes in intestinal permeability and absorption function [12].

Our aim in this study is to investigate the possible dysfunction in the zonulin-tight junction mechanism and also possible role of oxidative stress markers as malondialdehyde (MDA), lipid hydroperoxide (LOOH), Cu, Zn-superoxide dismutase (Cu-Zn-SOD) and glutathione (GSH) in acute pancreatitis. In addition, possible changes in serum zonulin levels in the early stages of these diseases may bring clinical use as a biomarker. Level changes in later stages may guide the follow-up of the disease in chronic stages.

Material and Methods

Ethical Statement

This study was approved by the Yeditepe University Animal Experiments Local Ethics Committee (Approval no: 2019/10-3).

Animals

Care and handling of the animals was in accordance with the Helsinki Declaration of 1975, as revised in 2000. Animals were housed in individual cages in a temperature-controlled room (23±1°C) and a light-dark cycle-controlled environment (12 h) with free access to food and water. Experiments were performed on 21 Sprague-Dawley rats with an average body weight of 250-320 g.

Experimental Design

In this study, a total of 21 rats, 7 in each group, were used. Groups were determined as group 1 (control, sham laparotomy), group 2 (acute pancreatitis (AP), 48% ethyl alcohol), and group 3 (severe pancreatitis (SP), 80% ethyl alcohol). AP and SP were performed adhering to the experimental model previously created by us [13]. Our study was carried out in accordance with the Animal Care and Use Committee (ACUC) criteria. Animals were fed with standard lab diet and *ad libitum* water before and after surgery. Animals had access to standard laboratory feed and water *ad libitum* and were not subjected to any restrictions.

Surgical Procedure

After anesthesia, 48% ethyl alcohol was injected into one group at a dose of 1 cm^3 with a fine dental needle into the suspended biliopancreatic duct to be opened with a midline incision after anesthesia, and 80% ethyl alcohol was injected into the other group. All groups were sacrificed on the 3rd postoperative day. Maximum blood was taken by abdominal midline exploration and cardiac puncture under anesthesia, and in all groups, the 5-6 cm segment of pancreatic tissues, distal ileum and transverse colon were excised. The tissues were divided into two, fixed in 10% buffered formalin, embedded in paraffin for standard histologic examinations and immediately frozen at -80°C for biochemical analysis.

Tissue Preparation and Biochemical Analysis

The intestinal tissue sample was diluted to a 20% w/v solution

in 20 mM ice-cold Tris HCl (pH 7.4) and homogenized with a Bosch Scintilla SA (Switzerland). The homogenate was centrifuged at 5000×*g* for 10 min; all biochemical parameters were determined in the same supernatant fraction of each homogenized pancreatic sample.

Assay of Zonulin

Determination of zonulin levels in intestinal tissue and serum samples were done using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique (Sunred Biological Technology®, Shanghai, China) according to the manufacturer's protocol. Assay range and sensitivity for zonulin levels were 0.3-90 ng/mL and 0.287 ng/mL, respectively.

Assay of Malondialdehyde (MDA)

One of the end products of lipid peroxidation is MDA. MDA levels were determined as previously described by Ohkawa et al.[14] with a minor modification. The intra- and inter-assay CV values were 3.1% (n=20) and 4.0% (n=20), respectively.

Assay of Lipid Hydroperoxide (LOOH)

LOOH levels were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange version 2 (FOX2) [15]. The intra- and inter-assay CV values were 3.5% (n=20) and 4.3% (n=20), respectively.

Assay of Cu, Zn, Superoxide Dismutase (Cu, Zn-SOD)

Cu, Zn-SOD activity were determined in terms of the inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine/xanthine oxidase used as a superoxide generator [16]. The intra- and inter-assay CV values were 3.6% (n=20) and 4.8% (n=20), respectively.

Assay of Glutathione (GSH)

Intestinal tissue GSH levels were measured as per the method of Beutler et al.[17]. The intra- and inter-assay CV values were 3.2% (n=20) and 4.4% (n=20), respectively.

Histopathological Examination

Tissue samples were routinely %10 formalin-fixed and paraffin-embedded. For histological evaluation each 5 µm section prepared and stained with H&E in Health Sciences University Haydarpasa Numune Training and Research Hospital Pathology Laboratory.

Statistical Analysis

Results of biochemical parameters were expressed as mean ± standard deviation. For comparing three groups comparison, one-way ANOVA test and post-hoc Tukey test were applied. Relationships between variables were assessed with Pearson's correlation coefficient. A P value equal to or lower than 0.05 was considered statistically significant. All analyzes were performed with the SPSS 22.0 (IBM Corp., Armank, USA) statistical package program.

Results

Biochemical Findings

Oxidative stress parameters (MDA, LOOH, Cu, Zn-SOD and GSH) and zonulin levels were examined in intestinal tissue and serum samples belonging to the experimental groups. Biochemical results in control, AP and SP groups were shown in *Table 1*.

In correlation analysis, it was found that the correlations in both AP and SP groups were largely in the same direction. Correlations of biochemical parameters in AP and SP were documented in *Table 2* and *Table 3*.

Histopathological Findings

As a result of histopathological examinations of pancreatic tissues belonging to the control groups, it was found that there were densely packed pancreatic acinar glands

Table 1. *Biochemical results in control, acute and severe pancreatitis group*

Parameters		Control (n=7)	Acute pancreatitis (n=7)	Severe pancreatitis (n=7)
		Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Tissue	MDA (µmol/g wet tissue)	57.31±8.46	68.28±6.64	72.71±9.62 a**
	LOOH (nmol/g wet tissue)	2.14±0.42	2.62±0.26 a*	2.88±0.32 a**
	Cu-Zn SOD (U/g wet tissue)	28.80±2.77	24.35±3.75	20.00±3.54 a***
	GSH (µmol/g wet tissue)	66.36±5.44	60.96±4.49	54.74±5.26 a***
	Zonulin (ng/g wet tissue)	3.38±0.84	11.37±1.76 a***	14.66±3.02 a***, b*
Serum	MDA (µmol/mL)	3.17±0.37	3.53±0.45	3.95±0.45 a**
	LOOH (nmol/mL)	0.47±0.08	0.58±0.09	0.76±0.13 a***, b*
	Cu-Zn SOD (U/mL)	20.16±2.49	17.53±1.99	16.27±2.05 a**
	Zonulin (ng/mL)	13.84±2.11	17.55±2.73 a*	20.47±2.37 a***, b*

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase*; ***GSH:*** *Glutathione*
** P<0.05, ** P<0.01, *** P<0.001;* ***a:*** *Compared to Control,* ***b:*** *Compared to Acute pancreatitis*

Table 2. *Correlations of biochemical parameters in acute pancreatitis*

Parameters		Tissue LOOH	Tissue Cu, Zn-SOD	Tissue GSH	Tissue Zonulin	Serum MDA	Serum LOOH	Serum Cu, Zn-SOD	Serum Zonulin
Tissue MDA	r	0.926**	-0.865*	-0.860*	0.810*	0.935**	0.929**	-0.912**	0.815*
	p	0.003	0.012	0.013	0.027	0.002	0.003	0.004	0.025
Tissue LOOH	r	-	-0.861*	-0.945**	0.752	0.904**	0.855*	-0.918**	0.761*
	p	-	0.013	0.001	0.051	0.005	0.014	0.004	0.047
Tissue Cu, Zn-SOD	r		-	0.907**	-0.940**	-0.887**	-0.976**	0.938**	-0.972**
	p		-	0.005	0.002	0.008	0.000	0.002	0.000
Tissue GSH	r			-	-0.823*	-0.820*	-0.869*	0.872*	-0.816*
	p			-	0.023	0.024	0.011	0.010	0.025
Tissue Zonulin	r				-	0.799*	0.926**	-0.884**	0.976**
	p				-	0.031	0.003	0.008	0.000
Serum MDA	r					-	0.910**	-0.898**	0.819*
	p					-	0.004	0.006	0.024
Serum LOOH	r						-	-0.941**	0.960**
	p						-	0.002	0.001
Serum Cu, Zn-SOD	r							-	-0.920**
	p							-	0.003

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase;* ***GSH:*** *Glutathione; * P<0.05 ** P<0.01 *** P<0.001*

Table 3. *Correlations of biochemical parameters in severe pancreatitis*

Parameters		Tissue LOOH	Tissue Cu, Zn-SOD	Tissue GSH	Tissue Zonulin	Serum MDA	Serum LOOH	Serum Cu, Zn-SOD	Serum Zonulin
Tissue MDA	r	0.929**	-0.773*	-0.976**	0.722	0.831*	0.962**	-0.935**	0.798*
	p	0.002	0.041	0.000	0.067	0.020	0.001	0.002	0.031
Tissue LOOH	r	-	-0.673	-0.939**	0.699	0.838*	0.899**	-0.759*	0.809*
	p	-	0.097	0.002	0.081	0.019	0.006	0.048	0.028
Tissue Cu, Zn-SOD	r		-	0.816*	-0.939**	-0.904**	-0.792*	0.764*	-0.930**
	p		-	0.025	0.002	0.005	0.034	0.045	0.002
Tissue GSH	r			-	-0.822*	-0.920**	-0.985**	0.910**	-0.888**
	p			-	0.023	0.003	0.000	0.004	0.008
Tissue Zonulin	r				-	0.940**	0.814*	-0.692	0.979**
	p				-	0.002	0.026	0.085	0.000
Serum MDA	r					-	0.879**	-0.764*	0.982**
	p					-	0.009	0.046	0.000
Serum LOOH	r						-	-0.918**	0.863*
	p						-	0.004	0.012
Serum Cu, Zn-SOD	r							-	-0.718
	p							-	0.069

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase;* ***GSH:*** *Glutathione; * P<0.05 ** P<0.01 *** P<0.001*

(Fig. 1); in the AP group, focal segmental fibrosis among intact parenchymal areas were selected. Plasma cell-predominant moderate inflammatory cell infiltration in which rare eosinophil leukocytes and ductal proliferation, disorganization, irregularity was observed in the fibrosis area in the pancreatic ducts. Regenerative changes were also detected in the ductal epithelium *(Fig. 2)*. In the SP group, distinct acinar atrophy, extensive fibrosis, hemorrhage, intense inflammation which rich in polymorphous leukocytes, fat necrosis and acinar necrosis were seen in the parenchyma *(Fig. 3)*.

As a result of the histopathological examinations of the intestinal tissues, evenly distributed villi on the luminal surface and mucosal layers of usual morphology were observed in the control group *(Fig. 4)*. In the AP group,

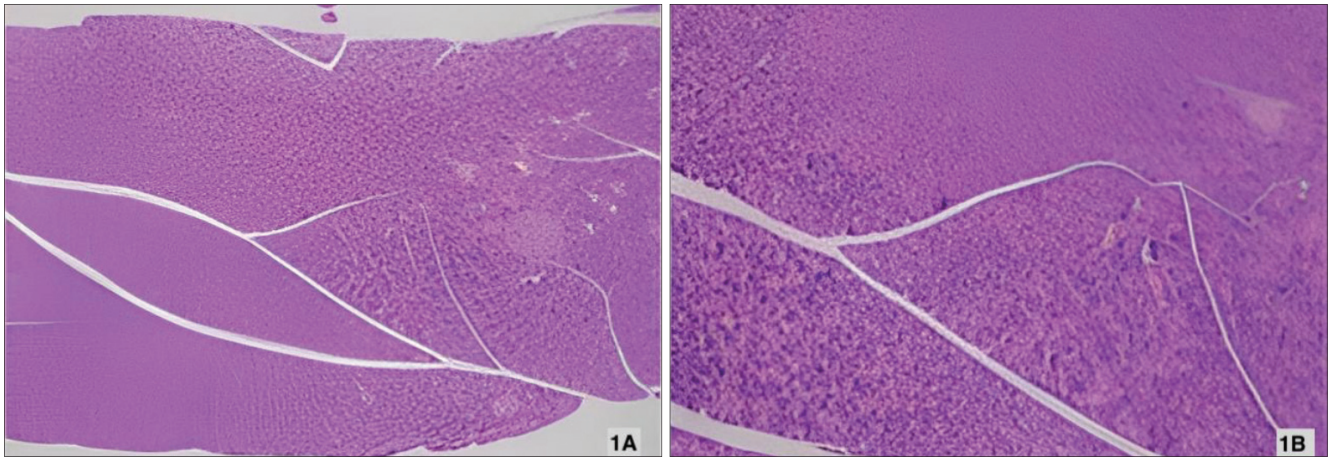

Fig 1. Pancreatic tissue of the control group; **A.** Densely packed pancreatic acini (H&E X200) **B.** Normally pancreatic tubuloacinar glands and 1 Langerhans Islet (H&E X40) in the right midline

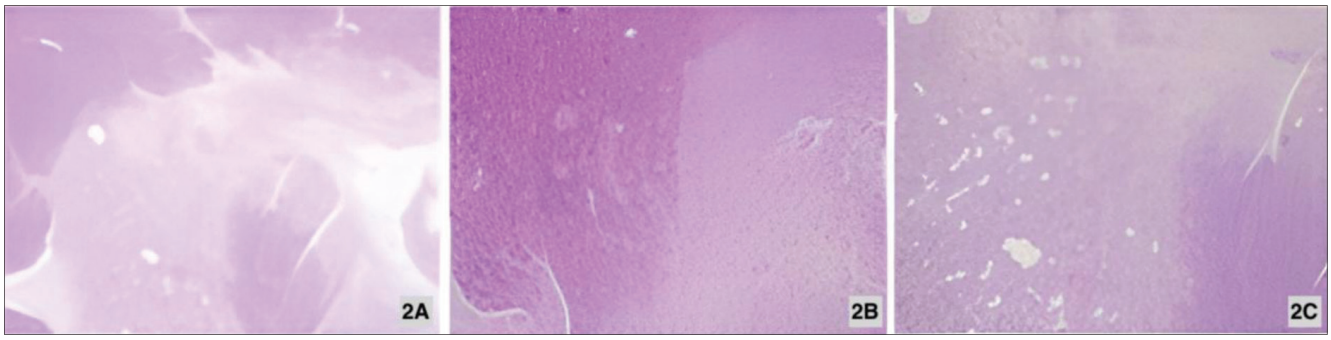

Fig 2. Pancreatic tissue of the acute pancreatitis group; **A.** Focal segmental fibrosis between intact parenchymal areas, moderate inflammatory cell infiltration, and irregularity of the pancreatic ducts (H&E X40) **B.** Plasma cell predominant chronic inflammation in which sparse eosinophil leukocytes are selected in the fibrosis area (H&E X200). **C.** Periductal fibrosis and moderate chronic inflammation. Proliferation, disorganization and irregularity in the ducts. Regenerative changes in the ductal epithelium (H&E X200)

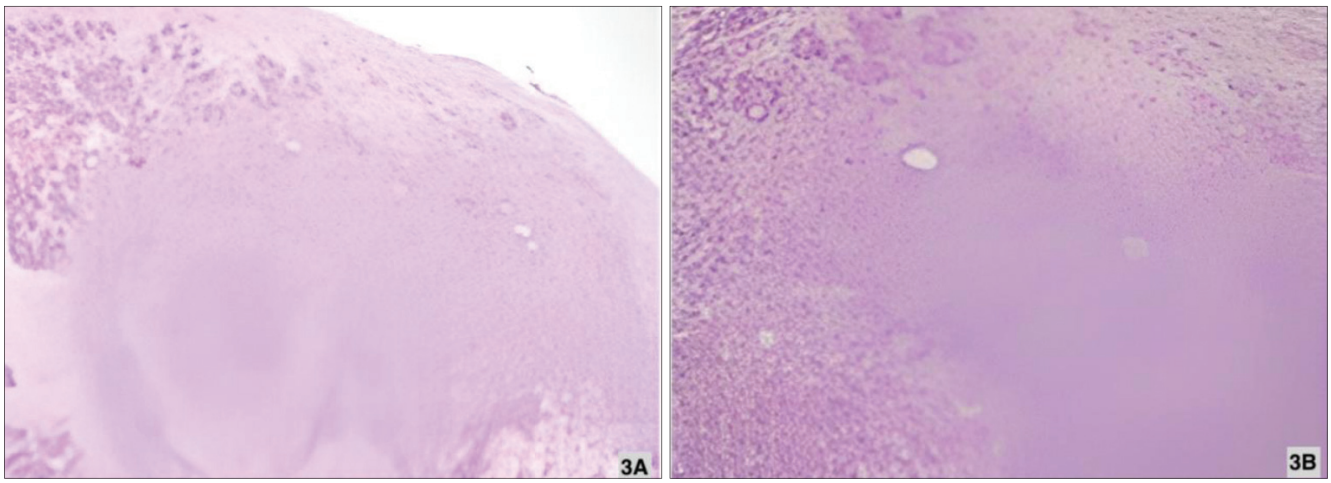

Fig 3. Pancreatic tissue of the severe pancreatitis group; **A.** Significant acinar atrophy, extensive fibrosis and intense inflammation in the parenchyma. Fat necrosis (lower right) and acinar necrosis (middle) (H&E X40). **B.** Intraparenchymal extensive fibrosis, intense inflammation rich in polymorphous leukocytes and hemorrhage (H&E X400)

moderately blunting of the villus size, moderate degeneration and desquamation in the intestinal epithelium and loss of intestinal epithelial cells, irregularity and disorganization in the crypts in the lamina propria were detected. Mild inflammatory cell increase accompanied by eosinophil leukocytes in the lamina propria was observed *(Fig. 5)*. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected *(Fig. 6)*.

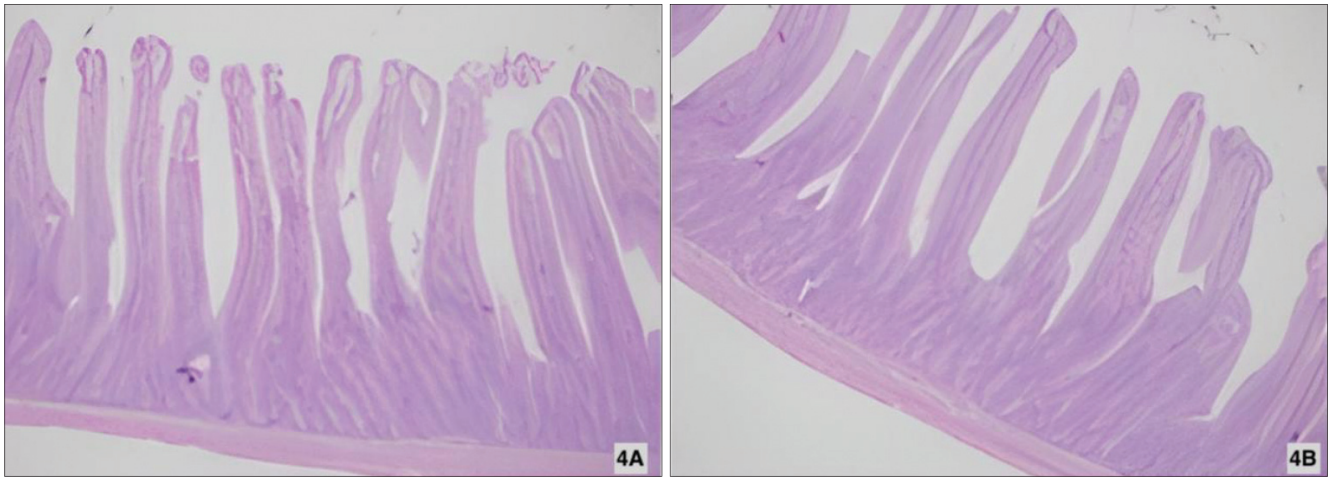

Fig 4. Intestinal tissue of the control group; **A.** Regularly distributed villus on the luminal surface and mucosal layers of usual morphology (H&E X200). **B.** Villus that are parallel to each other and distributed regularly (H&E X200)

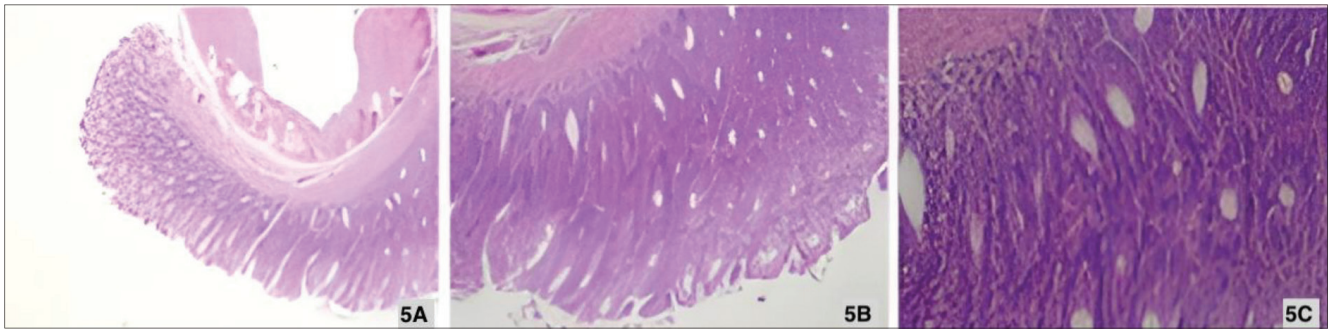

Fig 5. Intestinal tissue of the acute pancreatitis group; **A.** Moderate blunting in villus sizes and loss of intestinal epithelial cells (H&E X100). **B.** Moderate degeneration and desquamation of the intestinal epithelium. Irregularity in the crypts in the lamina propria (H&E X200). **C.** Disorganization in crypts and mild inflammatory cell increase in lamina propria accompanied by eosinophil leukocytes (H&E X400)

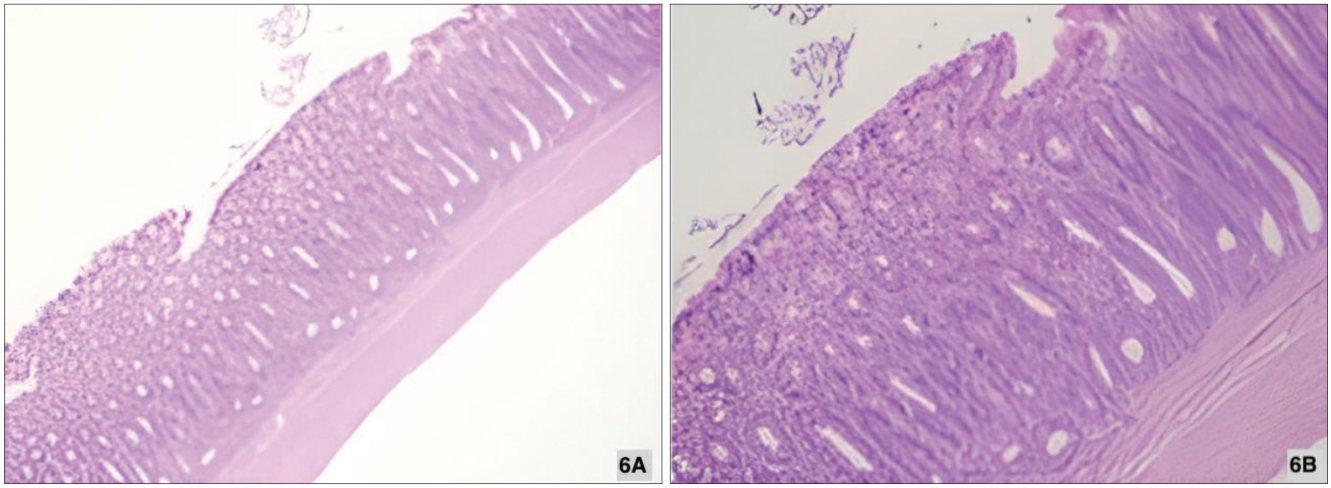

Fig 6. Intestinal tissue of the severe pancreatitis group; **A.** Completely flattened mucosal surface and severe villi loss (total villous atrophy). Crypt hyperplasia in the lamina propria (H&E X200). **B.** Severe villus atrophy on the surface. Disorganization and hyperplasia of the crypts in the lamina propria (H&E X400)

Discussion

ROS and reactive nitrogen species (RNS), which play an active role in the early and late stages of AP, cause deterioration in cell membrane and functions by direct action on lipids and proteins, and damage in pancreatic cells with the release of lysosomal enzymes in experimental and human studies [18-20]. However, to date no information about the possible dysfunction in the zonulin-tight junction mechanism in AP has been reported. The main findings of this study were that (i) in the AP group, while LOOH and zonulin levels in intestinal tissues were significantly

higher than control, only zonulin levels in serum samples were found higher than control, (ii) while zonulin levels in intestinal tissue samples of the SP group were found to be significantly higher compared to the AP group, in serum samples, only LOOH levels were found to be significantly higher, (iii) the serum zonulin could be used to distinguish AP from SP was serum zonulin. Zonulin is basically involved in the pathogenesis of pancreatitis. Patients with pancreatitis may be more exposed to impaired gut barrier function [21]. Zonulin can be used as a biomarker of impaired gut barrier function for pancreatitis and can be a potential therapeutic target for the treatment of these disturbing situations.

Lipid peroxidation and free oxygen radicals may also be effective in the pathogenesis of AP as a result of oxidative stress. In experimental pancreatitis studies, while MDA levels, the final product of lipid peroxidation, increased, it was observed that glutathione peroxidase (GPaz), catalase, and SOD, which are protective enzymes from free oxygen radicals and GSH decreased [19-21]. In experimental pancreatitis studies, while MDA levels, the final product of lipid peroxidation increased, glutathione peroxidase (GPaz), catalase and SOD, which are protective enzymes from free oxygen radicals, and GSH were found to decrease [22-27]. Intestinal epithelial barrier dysfunction and increased permeability have been described in many human diseases, including SP [28-33]. In current study, while LOOH and zonulin levels increased in AP compared to control group, it was found to be increased in SP group compared to AP in pancreas tissue. Serum zonulin levels increased significantly in AP compared to the control group, while it was found to be increased in SP compared to AP. Moreover, a positive correlation was found between serum zonulin and tissue zonulin, tissue and serum MDA, and serum LOOH. The increase in tissue and serum zonulin levels in the study confirms the increase in intestinal permeability in AP. While there was a negative correlation between tissue zonulin and tissue GSH and Cu, Zn-SOD activity, a negative correlation was found between serum zonulin and tissue and serum Cu, Zn-SOD, and tissue GSH in AP. It indicates that oxidative stress increases in AP and a decrease in antioxidant capacity eliminates the increased levels of free radicals. The impairment of intestinal permeability in pancreatitis was demonstrated by the increase of oxidant substances such as LOOH and MDA, and the decrease of antioxidants such as GSH and Cu, Zn-SOD. All of results support the hypothesis of increased oxidative stress and decreased antioxidant capacity during increased intestinal permeability with increased zonulin levels with AP. Zonulin secretion seems to be one of the most important causes of increased intestinal permeability in AP and SP. Fishman et al.[34] demonstrated that damage to the unstirred mucus layer with evidence of oxidative stress occurs during AP-induced gut barrier failure in rats. Liu et al.[28] suggests that gut mucosal damage takes place in early phase of AP and more severe in SP than mild AP patients. The results of our study are supported by the results of Fishman et al.[34]'s study.

In the SP group, distinct acinar atrophy, extensive fibrosis, hemorrhage, intense inflammation which rich in polymorphous leukocytes, fat necrosis and acinar necrosis were seen in the parenchyma in pancreatic tissues. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected in intestinal tissue. Our biochemical findings are also supported by histopathological improvement in pancreatic and intestinal tissues. These results suggest that breakdown of intestinal mucosa via intense inflammation and necrosis may increase in intestinal permeability in AP and SP.

The severity of disease, high zonulin, LOOH, and MDA, low GSH and Cu, Zn-SOD activity are associated with increased intestinal permeability in early phase of AP. The inflammatory signaling and response in pancreatitis is mediated in part through ROS as important mediators of oxidative stress. Serum zonulin levels may be a promising clinical marker for differentiation AP and SP in clinical practice. Patients with pancreatitis may be more exposed to impaired gut barrier function. Serum zonulin levels can be used in the evaluation of intestinal permeability in acute pancreatitis. In order to use our results in clinical practice, clinical studies showing the relationship between impaired intestinal permeability in pancreatitis and zonulin are also required.

Availability of Data and Materials

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

Financial Support

There is no funding source.

Competing Interest

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

Author Contributions

AY, BPK, AT, RG, EU and HU conceived and supervised the study. AY, SY, SD and ZKS collected and analyzed data. SD made laboratory measurements. ZKS 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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Research Article

Lyophilized Extender Supplemented with Rainbow Trout *(Oncorhynchus mykiss)* Seminal Plasma Improves Cryopreservation of Ram Sperm

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Abstract: This study aimed to investigate the effect of the Rainbow trout seminal plasma (RTSP) supplemented (control, 10% or 15%) lyophilized extender on freezability of ram semen. Collected semen was pooled and split into two aliquots, and each of the ejaculates was diluted with fresh or lyophilized extenders with RTSP (0%, FC and LC; 10% F10 and L10or 15% F15 and L15) using two-step dilution method. Semen was frozen using the programmable freezing machine. Semen samples were examined for sperm motility, defective acrosomes, plasma membrane integrity and DNA fragmentation at native and post-thaw stage. The highest percentages of post-thaw motility and plasma integrity were observed in the F10, F15, and L15 ($P<0.05$). There was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control. The highest percentage of post-thaw DNA fragmentation values were observed in the FC group, while the lowest DNA fragmentation was obtained in the F15 but only significant different from FC and LC groups. In conclusion, the findings of this study show that the lyophilized extender with 15% RTSP added can be used successfully for freezing ram semen.

Keywords: *Cryopreservation, Lyophilized extender, Rainbow trout seminal plasma, Ram semen*

Gökkuşağı Alabalığı *(Oncorhynchus mykiss)* Seminal Plazması İlave Edilmiş Liyofilize Sulandırıcı, Koç Spermasının Dondurulmasını İyileştirir

Öz: Bu çalışma Gökkuşağı alabalığı seminal plazması (ASP) ilave edilmiş (kontrol, %10 ve %15) liyofilize sulandırıcının koç spermasının dondurulabilirliği üzerindeki etkisini belirlemeyi amaçlamıştır. Alınan sperma birleştirilerek, iki eşit hacime bölündü ve ejakülatların her biri, iki aşamalı sulandırma yöntemi kullanılarak ASP (%0, FC ve LC; %10 F10 ve L10 veya %15 F15 ve L15) ilave edilen taze ve liyofilize sulandırıcılarla sulandırıldı. Sperma programlanabilir dondurma makinesi kullanılarak donduruldu. Sperma numuneleri, taze ve eritme sonrası aşamada motilite, akrozom hasarı, plazma membran bütünlüğü ve DNA fragmentasyonu bakımından incelendi. Eritme sonrası en yüksek motilite ve plazma membrane bütünlüğü F10, F15 ve L15 gruplarında gözlendi ($P<0.05$). Eritme sonrası motilite değerlerine paralel olarak %10 ASP içeren liyofilize sulandırıcının plazma membran bütünlüğü, kontrol gruplarından daha yüksek bulundu. Sulandırıcı grupları kontrol grupları ile karşılaştırıldığında, eritme sonrası akrozom bozukluğu bakımından istatistiksel bir fark tespit edilmedi. Eritme sonrası DNA fragmentasyonu en yüksek FC grubunda gözlenirken, en düşük F15 grubunda elde edildi fakat yalnızca FC ve LC gruplarından istatistiksel olarak farklıydı ($P<0.05$). Sonuç olarak, bu araştırmanın bulguları %15 ASP ilave edilen liyofilize sulandırıcının koç spermasının dondurulmasında başarıyla kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Dondurarak saklama, Gökkuşağı alabalığı seminal plazması, Koç sperması, Liyofilize sulandırıcı*

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Introduction

Although researchers have tried to freeze ram semen with different protocols and diluents for years, the fertility rates obtained with frozen semen are not as high as those acquired with fresh semen [1,2]. The reason for the reduction in fertilization rate of ram sperm is the decrease in motility, increase in acrosomal damage, and adversely affecting chromatin integrity in the post-thaw stage [3-6].

Increased levels of reactive oxygen species (ROS) due to cold shock origin changes in cellular functions and increase the susceptibility of semen to oxidative damage [7]. Thus, the migration ability of frozen-thawed ram semen in the female genital tract decreases, and the fertility rates after cervical insemination are not at the desired level [8,9].

Studies to ameliorate the maintenance of ram semen are concentrated on modifying extenders by adding various components to preserve sperm motility, membrane integrity, and fertilization capacity [3,10,11].

Previous studies of minimizing the effect of cryo-damage on spermatozoa have dealt with the addition of various complements such as seminal plasma of the same or different species in the freezing extenders or thawing solutions [12-14]. Seminal plasma comprises notable biochemical constituents that are responsible for regulating sperm function [15]. Supplementation of rainbow trout seminal plasma (RTSP) to egg-yolk-based extenders used cryopreservation of ram and goat semen successfully [13,16]. Unlike mammals, since Rainbow trout do not have accessory glands, RTSP does not originate from the accessory reproductive gland but the blood plasma and cuboidal cells in the semen duct [17]. Monosaccharides and triglycerides in RTSP form the energy source of spermatozoa and maintain the viability of these cells [18]. RTSP proteins, on the other hand, are essential in terms of helping the motility ability and maintaining the viability of the spermatozoon [19]. Shaliutina-Kolešová et al.[20] reported that about 25% of the proteins identified in the RTSP are multifunctional proteins (apolipoprotein, transferrin, serum albumin), immune response-related proteins (complements), antioxidant proteins (SOD, glutathione-S-transferase, thioredoxin, etc.), cold-shock proteins, enzymes (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, creatine kinase) and 14-3-3 family members. At the same time, some specific proteins, such as type-4 ice-formatting protein, known as LS-12 anti-freeze protein, isolated in RTSP prevent the freezing of fish semen in low-temperature waters [17,21]. Due to all these properties of rainbow trout seminal plasma, it has been commented that adding it to the extender positively affects the freezability of ram semen [13].

Lyophilization is mainly used to remove the water from sensitive biological origin products without any damage. Thus these sensitive products can be preserved, permanently stored, and be reconstituted smoothly by water addition [22]. Chemicals, biotechnological products, bacteria, cells and tissues, diagnostic drugs, antibiotics, and vaccines are examples of products provided by lyophilization technology. Therefore, the extender supplemented with RTSP, which has proven successful in freezing ram semen, can be lyophilized and stored later in semen cryopreservation.

This study aimed to investigate the effect of the RTSP supplemented (control, 10% or 15%) lyophilized and fresh extender on post-thaw ram spermatological parameters (motility, plasma membrane, acrosome, and DNA integrities).

Material and Methods

Ethical Approval

The Scientific Ethical Committee (Bursa Uludag University, Bursa, Turkey, Approval No: 2020-05/03) accepted all protocols regarding the experimental design and evaluation procedures planned in the research.

Semen Extender Preparation

Rainbow trout milt was collected by abdominal massage using the method of Glogowski et al.[23] Subsequently, RTSP obtained from milt using the method of Ustuner et al.[13] was aliquoted and stored for up to 1 month at -20°C in the eppendorf tubes until added to the extender.

The extenders to be used in the experiment were designed to be fresh (control) and lyophilized. Both fresh and lyophilized extenders were prepared by a two-stage dilution method. Tris-based extenders were prepared by the addition of 20% egg yolk (EY), and Extender A did not contain glycerol. The freezing extender (Extender B) was composed of Extender A with added EDTA (0.15 g/100 mL), trehalose (3.8 g/100 mL), and glycerol 6% (v/v) [24]. In order to achieve successful lyophilization of the lyophilized group, glycerol was added to the lyophilized groups (extender B) after the lyophilization process.

RTSP's miscellaneous concentrations (0%, 10%, or 15%) were added to the groups of extender A and B with respect to the experimental design. Extenders (fresh (F) or lyophilized (L)) were designated as follows: FC, (no RTSP); F10, 10% RTSP; F15, 15% RTSP and LC (no RTSP); L10, 10% RTSP and L15, 15% RTSP.

Lyophilization of the extenders was performed according to the procedure previously described by Alcay et al.[25] After the preparation process, each extender group was lyophilized using Freeze-Dryer (Labconco, Kansas City, U.S.) Subsequently, prepared extenders were stored at 4°C until use.

Semen Collection, Evaluation, and Dilution

Three to 5 aged five rams from Bursa Uludag University,

Faculty of Veterinary Medicine in Bursa were selected for semen collection using electrically stimulated ejaculation method twice a week during the non-breeding season (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [3]. The semen collection process was repeated five times.

Collected semen was immediately evaluated and ejaculates with a thick consistency, a rapid wave motion (3-5 on a 0-5 scale), and >70% initial motility were pooled [13].

Pooled ejaculates were split into two aliquots, and each of the ejaculates was diluted to a ratio of 1:2 (semen/extender) with fresh or lyophilized extenders A (0%, 10% or 15% RTSP). Subsequently, cooled to 5°C within 1 h. The cooled semen groups were then diluted to a 1:1 (semen/extender) ratio with fresh and lyophilized extenders B (5°C), respectively. Equilibration was performed for diluted semen samples at 2 h at 5°C.

Semen Freezing and Thawing

Equilibrated semen was placed into 0.25 mL straws and frozen at 3°C/min from +5 to -8°C and at 15°C/min from -8 to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France) (0.25 mL straw contained $125x10^6$ spermatozoa). The frozen straws were immersed in liquid nitrogen (-196°C), where they were stored for at least one month. To perform post-thaw semen evaluations, leastwise three straws from each group were thawed at 37°C for 30 sec in the water bath.

Semen Evaluation

All semen parameters were assessed at the following two-time points: native and post-thaw stages.

Semen freezing and evaluation of semen parameters were performed by the same person throughout the study. Sperm motility was assessed by using a heated mechanical stage attached phase-contrast microscope (Olympus BX51) subjectively [26].

Fluorescein Lectin Staining Assay (FITC Conjugated *Pisum sativum* Agglutinin (FITC-PSA)

Assessment of acrosome integrity was performed using the method of Ustuner et al.[14] According to the procedure, stained slides were assessed under a fluorescent microscope, and at least 200 spermatozoa were counted for evaluation.

The Hypoosmotic Swelling Test (HOST)

Evaluation of the functional integrity of the sperm membrane was performed by incubating 10 µL of semen with 100 µL of a 100 mOsM hypoosmotic solution. After incubation at 37°C for 60 min., 200 spermatozoa were assessed under phase-contrast microscopy, and coiled or swollen tails were noted [27].

TUNEL (Terminal-Deoxynucleotidyl-Transferase-Mediated-dUTP Nick-End Labelling) Assay

For the assessment of DNA integrity, the manufacturer's slight-modified TUNEL technique was performed [3]. After the labeling process, slides were analyzed via fluorescence microscopy, and leastwise 100 spermatozoa were assessed to determine the TUNEL positive sperm percentage.

Statistical Analysis

All data obtained from the study were analyzed using SPSS. (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Shapiro Wilk test was used as a normality test. According to normality and homogeneity tests, the data which present normal distribution was analyzed using One Way Anova Test, and Tukey was used as a Posthoc test. The semen parameters presented nonparametric distribution was analyzed using the Kruskal Wallis test followed by the Mann Whitney U test.

Results

Motility, plasma membrane integrity, defected acrosome and DNA fragmentation rates of pooled fresh semen samples were 77±2.7%, 85.2±3.2%, 14.4±5.2%, and 2.2±0.8%, respectively.

The effects of the fresh and lyophilized diluents containing different concentrations of RTSP at post-thaw on ram sperm motility, plasma membrane, acrosome, and DNA integrity are presented in *Table 1*.

The freeze-thaw process diminished sperm motility

Table 1. *Mean motility, plasma membrane integrity, defected acrosome and DNA fragmentation rates in post-thawed ram semen. (mean±SEM)*

Groups	n	Motility (%)	Plasma Membrane İntegrity (%)	Defected Acrosome (%)	DNA Fragmentation (%)
FC	15	44.29±1.56^{a}	70.40±2.38^{a}	39.8±3.02	13.0±0.71^{a}
F10%	15	52.67±1.28^{b}	75.6±1.36^{b}	36.40±6.08	9.6±0.51^{bc}
F15%	15	54.23±1.59^{b}	76.00±4.32^{b}	38.00±4.38	8.80±0.38^{c}
LC	15	41.07±1.83^{a}	63.20±6.17^{a}	36.60±3.03	10.60±0.75^{b}
L10%	15	46.15±1.29^{ac}	68.00±3.90^{a}	34.00±1.92	9.00±0.45^{bc}
L15%	15	51.67±1.42^{bc}	76.80±3.22^{b}	38.60±3.56	10.00±0.90^{bc}

^{a,b,c} *Different superscripts within the same column are significantly different (P<0.05)*

progressively (P<0.05). The highest percentages of post-thaw motility were observed in the F10, F15, and L15, and these rates were significantly higher than observed in the control groups (P<0.05). It was observed that the motility of the L10 group was numerically higher than the FC and LC groups, although not statistically (P>0.05).

The plasma membrane functional integrity was affected by the freeze-thaw process negatively (P<0.05). Higher percentages of post-thaw plasma integrity were observed in the F10, F15, and L15 groups than in the other groups (P<0.05). In parallel with the post-thawing motility values, the plasma membrane integrity of the lyophilized diluent containing 10% RTSP was found to be slightly higher than the LC group.

Acrosome integrity deteriorated during the freeze-thaw process but there was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control (P>0.05).

Sperm DNA integrity was negatively affected by freeze-thaw process. The highest percentage of post-thaw DNA fragmentation values were observed in the FC group (P<0.05). Although the lowest DNA fragmentation was obtained in the F15 group, it was only statistically different from the control groups of fresh and lyophilized extenders (P<0.05).

Discussion

Although cattle and pigs are the most commonly used livestock of artificial insemination biotechnology world-wide; In sheep farming, since acceptable pregnancy rates cannot be obtained with frozen semen, artificial insemination practices cannot be utilized to the same extent. The most important reason is that ram semen is much more sensitive to freezing processes than other farm animals. In many studies conducted in recent years, it has been revealed that seminal plasma proteins have various effects on the freezing of semen [12-14,28]. The current study, therefore, set out to assess the effect of the lyophilized extender with added rainbow trout seminal plasma on ram sperm freezability.

The freeze-thaw process of semen causes irreversible damage to motility, acrosome, plasma membrane, and DNA integrity [3,24,29]. Comparing fresh pooled semen to post-thaw results of the current study, predictably acrosome, plasma membrane, and DNA integrities were affected by the cryopreservation process negatively (P<0.05).

Spermatozoa must have motility to progress through the female genital canal to reach the fertilization area and penetrate the zona pellucida (ZP) with the cumulus cells on the oocyte surface [30]. Sperm motility is affected by many factors such as the content and osmotic pressure of the extender used in the cryopreservation process, cooling rate, cold shock, freezing rate, presence of ROS, thawing temperature, and time. In the current study, post-thaw motility of the lyophilized-extender supplemented with 15% RTSP and freshly prepared extender groups with 10% and 15% RTSP (P<0.05) were statistically higher than the control groups. The positive effect of RTSP on the post-thaw success of ram semen of lyophilized extender was in good agreement with Ustuner et al.[13] and Alcay et al.[16] This positive effect is probably due to the presence of cathepsin D and M, calpain, cytosolic nonspecific dipeptidase, proteasome, and antifreeze protein (type-4 ice-structuring protein [LS-12]) in RTSP. Among the mentioned proteins, especially LS-12 is known to prevent fish semen from being damaged in cold water conditions, and notably it is not found in mammalian semen [17,21]. It is known that other proteins mentioned above are important in stimulating motility and eliminating metabolic wastes of damaged spermatozoa [31]. However, it was observed that the post-thaw motility of the 10% RTSP supplemented lyophilized group was similar to the control groups. This suggests the possibility that RTSP may be inactivated by lyophilization when used at low rates.

The ability of spermatozoa to exchange substances with the external environment is necessary for its physiology. This phenomenon is only possible if the plasma membrane functional integrity is preserved [32]. During cryopreservation, cold shock, osmotic pressure change, ice crystals, and ROS formation cause lipid composition changes in the cell membrane, resulting in morphological damage and loss of function in the plasma membrane [33]. Nur et al.[3] reported that there is a correlation between sperm motility and functional plasma membrane integrity. In our study, it was observed that the plasma membrane integrity of this study groups was parallel and associated with motility. Furthermore, the post-thaw functional sperm plasma membrane integrity in lyophilized extender supplemented with added RTSP was found higher than the findings of Alcay et al.[25].

As mentioned in the literature reviews, acrosome reactions or premature capacitation are induced by cryo-preservation [34]. The acrosome defect of 35-65% caused by the freeze-thaw process reported in several previous studies is consistent with the data obtained in the study [13,29,35,36]. There was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control.

The protection of the physiological and morphological integrity of the paternal DNA, with its compact structure, is of great importance for the formation and development of the embryo [37]. In spermatozoa, minimal DNA damage is reversible and does not pose a significant threat to

fertilization, while severe DNA damage causes sterility [38]. This negative effect of DNA damage on fertility is essential in interrupting the viability and development of the embryo rather than reducing the fertilization ability of the sperm [39]. Ram spermatozoa are more susceptible to DNA damage during the thawing process after cryopreservation compared to other species [3,26]. Therefore, the evaluation of DNA integrity is esteemed to determine post-thaw semen quality. The DNA fragmentation rates obtained in the current study were consistent with previously reported results by Alcay et al.[25] The highest percentage of post-thaw DNA damage values were observed in the FC group ($P<0.05$). However, the DNA fragmentation rate of the LC group was similar to all groups except for FC and F15 extender groups ($P<0.05$). Generally, according to the current study data, we can infer that the lyophilized group conserved DNA integrity near the freshly prepared group against damage due to cryopreservation ($P>0.05$).

Sheep artificial insemination on the field commonly is depend on the successfully cryopreserved ram semen. It is an advantage that the lyophilization of this extender is more practical than the extender that is prepared fresh daily.

As a result of the current study, it was seen that there was no difference between the freshly prepared extender and the lyophilized extender with 15% RTSP added. Hereby, frozen semen will have the opportunity to be used more widely in the field.

Competing Interests

There is no conflict to interest.

Authors' Contributions

BU experimental design, prepared extender, collected and frozen semen, evaluated native and post thawed spermatological parameters, wrote the manuscript;, SA lyophilization of extender and contrubution to experimental design; EG staining of spermatological smears with fluorescent dyes, statistical evaluation of the findings; MMY semen collection with electro ejaculator and semen frozen and contrubution to preparation of semen extender; AA semen collection with electro ejaculator and semen frozen; OH evaluation of the manuscript in terms of English grammar and statistical evaluation of the findings; MD collected milt from Rainbow trout; NTO semen collection with electro ejaculator; EA the manuscript in terms of English grammar and statistical evaluation of the findings; ZN edited the manuscript scientifically.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the correspond-ing author (B. Üstüner).

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Research Article

A Comparative Study of the Nonlinear Methods for Estimating Body Weight Based on Body Measurements in Different Sample Sizes in Morkaraman Sheep

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Abstract: The objective of this study is to estimate the body weight of Morkaraman sheep from body measurements with nonlinear models. Five different models (allometric, logistic, saturation growth, exponential and incomplete gamma) are used to estimate best-fitted model for relationship between body length and body weight of Morkaraman sheeps at different sample sizes. Selected 110 sheeps 3-5 years were scored for body weight, body length, height at wither, chest width and rump width. For determining the relationships with body weight between body measurements, correlation analysis was performed. The results of the correlation analysis indicated that the highest relationship according to all sample sizes was between body weight and body length (0.95, 0.90, 0.83, 0.81). Considering all parameters included in the model, the parameter showing the highest correlation with body weight was determined as body length according to all sample sizes. The highest correlation was found in 50 sample sizes (r=0.95). According to the small sample sizes (10-20), Logistic and Saturation growth models can be used to determine the body weight by using body length, on the other hand, the Incomplete gamma model is more successful to estimate body weight when the sample size is nearly 30 and 50.

Keywords: *Body measurements, Morkaraman, Logistic, Saturation growth, Incomplete gamma*

Morkaraman Koyunlarında Farklı Örnek Büyüklüklerinde Vücut Ölçülerine Göre Vücut Ağırlığının Tahmin Edilmesinde Doğrusal Olmayan Yöntemlerin Karşılaştırılması

Öz: Bu çalışmanın amacı, Morkaraman koyunlarının vücut ağırlığının lineer olmayan modellerle vücut ölçülerinden tahmin edilmesidir. Farklı örneklem büyüklüklerinde Morkaraman koyunlarının vücut uzunluğu ve vücut ağırlığı arasındaki ilişki için en uygun modeli tahmin etmek için beş farklı model (allometric, logistic, saturation growth, exponential and incomplete gamma) kullanılmıştır. Üç ile beş yaş arası seçilen 110 koyunun, vücut ağırlığı, vücut uzunluğu, cidago yüksekliği, göğüs genişliği ve sağrı genişliği değerleri ölçülmüştür. Vücut ölçüleri arasında vücut ağırlığı ile ilişkileri belirlemek için korelasyon analizi uygulanmıştır. Korelasyon analizi sonuçları, tüm örneklem büyüklüklerine göre en yüksek ilişkinin vücut uzunluğu ile (0.95, 0.90, 0.83, 0.81) vücut ağırlığı arasında olduğunu göstermiştir. Modelde yer alan tüm parametreler dikkate alındığında, örneklem büyüklüklerine göre vücut ağırlığı ile en yüksek korelasyonu gösteren parametre vücut uzunluğu olarak belirlenmiştir. En yüksek korelasyon 50 örneklem büyüklüğünde bulunmuş olup (r=0.95), küçük örneklem boyutlarına göre (10-20), vücut uzunluğunu kullanarak vücut ağırlığını belirlemek için Lojistik ve Saturation Growth modelleri, örnek büyüklüğü yaklaşık 30-50 arasında olanlarda ise vücut ağırlığını tahmin etmede Incomplete gama modeli daha etkili sonuçlar vermiştir.

Anahtar sözcükler: *Vücut ölçüleri, Morkaraman, Logistic, Saturation growth, Incomplete gamma*

Introduction

The increase in the number and size of cells in certain time intervals in accordance with the type of animal, shaped by the interaction of the genetic structure of living things and the environmental conditions in which they are found, is expressed as growth [1].

Macedo-Barragán [2] concluded that as an alternative to linear models, incomplete gamma and exponential models

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can be used to predict body weight of sheep using some body measurements.

Selection of the appropriate model requires a statistical decision process, since the live weight varies according to the species, environmental conditions and the trait studied. It has been reported in the literature that although a constant rate of weight gain occurs in certain periods for some characteristics of some living things, the weight increases in living things is not constant throughout their lifetime [3-5].

Linear models are often insufficient to model the growth of living things over the lifespan [4-6]. In the case of periods of different growth rates, it is useful or even necessary to use non-linear models, which are slightly more complex than linear models.

Tahtali et al.[7] aimed to model the body weight gains of Romanov lambs and individual growth curves using different equations. Cubic spline model, Logistic model, Gompertz model and Richard models were used as models in the study.

The determination and estimation of non-linear models are more difficult than linear models, and the results are determined iteratively using different methods [8].

In this study, it was tried to determine a model that gave the best fit between body weight and body measurements in different sample sizes. Although these measured variables can be explained by linear models, they can also be explained by nonlinear models. For this purpose, although the variables used in this study are in linear form, non-linear regression models can also be used for this purpose. In this study, it is aimed to determine nonlinear regression models as an alternative to linear regression models.

Material and Methods

Materials

The data used in this study were recorded on 110 Morkaraman sheep maintained in Atatürk University, Food and Livestock Research and Application Center. Body measurements of adult animals aged 3-5 years were recorded using a graduated measuring tape. Whole body measurements were taken with the animal standing, head up and weight on all fours without body movement. Body weight was taken using a suspended digital scale. Sheeps were included in the study as 10, 20, 30 and 50 separately according to sample size.

Methods

Correlation coefficients were used to determine the relationship between parameters. In addition, it is aimed to determine the best model according to the sample size in determining the live weight by using the nonlinear models.

The models were tested for goodness of fit by the (MSE) Mean Square Error and (R^2), adjusted coefficient of determination (R^2_{adj}), Akaike information criterion (AIC), Bayes information criterion (BIC) and mean squared prediction error (MEP). The statements of these evaluators are also presented in detail in Silveira et al.[9].

Results

Considering all parameters included in the model, the parameter showing the highest correlation with body weight was determined as body length according to all sample sizes. As indicated in *Table 1*, the highest correlation was found in 50 sample sizes (r=0.95). This was followed by sample sizes of 30, 20 and 10, respectively. The highest correlations for the BW parameters between BL were found 0.95, 0.90, 0.83 and 0.81, respectively. In addition, the lowest correlation values were between BW and HW (r=0.46), BW and HG (r=0.51) and, BW and RW (r=0.48). Considering all sample sizes, body length was included as an independent variable in nonlinear models.

Table 2 gives the results of nonlinear models, in which five different models for estimating best-fitted model for relationship between body length and body weight of Morkaraman sheeps at different sample sizes.

R^2 and MSE values for models estimated by five different models and sample sizes have been used to determine the best fit models.

Considering the sample sizes; the lowest R^2 and the highest MSE values occurred in the group with sample size 10. According to this group, the highest R^2 value (0.64) was

Table 1. *Correlations between body weight and body measurements with different sample sizes*

Body Weight	Sample Size	BL	HD	HW	CW	HG	RW
BW	10	0.81**	0.57*	0.46	0.73**	0.51	0.48
BW	20	0.83**	0.72*	0.58*	0.75**	0.66*	0.53
BW	30	0.90**	0.78**	0.60*	0.74**	0.76*	0.61*
BW	50	0.95**	0.81*	0.69*	0.74**	0.76*	0.66*

** P<0.01, * P<0.05; **BW:** Body weight, **BL:** Body length, **HW:** Height at wither, **CW:** Chest width, **RW:** Rump width

Table 2. *Estimation nonlinear models for predicting body weight of Morkaraman lambs from body length with different sample sizes*

n	Model	*Equation*	P	R^2	MSE
10	Allometric	$0.004BL^{2.09}$	0.035	0.59	15.18
	Logistic	$196.85/(1+\exp^{(3.446-0.0512BL)})$	0.020	0.64	14.98
	Saturation growth	-19.785BL/(-196.71+BL)	0.016	0.61	14.76
	Exponential	$5.0142\exp^{(0.0375BL)}$	0.020	0.60	14.91
	Incomplete gamma	$0.1014BL^{1.214}\exp^{(0.021BL)}$	0.028	0.63	14.89
20	Allometric	$0.003BL^{2.11}$	0.016	0.66	14.01
	Logistic	$202.45/(1+\exp^{(3.4141-0.0548BL)})$	0.013	0.65	14.01
	Saturation growth	-18.471BL/(-188.16+BL)	0.008	0.71	13.44
	Exponential	$4.7811\exp^{(0.0108BL)}$	0.002	0.60	13.75
	Incomplete gamma	$0.1008BL^{1.303}\exp^{(0.025BL)}$	0.008	0.68	13.67
30	Allometric	$0.003BL^{2.14}$	0.012	0.74	12.11
	Logistic	$201.48/(1+\exp^{(3.358-0.0442BL)})$	0.008	0.79	12.02
	Saturation growth	-20.016BL/(-198.34+BL)	0.010	0.78	12.16
	Exponential	$4.842\exp^{(0.0392BL)}$	0.002	0.79	12.08
	Incomplete gamma	$0.1021BL^{1.136}\exp^{(0.019BL)}$	0.001	0.82	11.88
50	Allometric	$0.003BL^{2.15}$	0.003	0.88	12.02
	Logistic	$199.61/(1+\exp^{(3.303-0.0398BL)})$	0.001	0.89	12.00
	Saturation growth	-20.038BL/(-199.16+BL)	0.001	0.86	12.01
	Exponential	$4.8805\exp^{(0.0384BL)}$	0.001	0.88	12.02
	Incomplete gamma	$0.1019BL^{1.149}\exp^{(0.020BL)}$	0.001	0.92	11.94

Table 3. *Estimation linear equations for predicting body weight of Morkaraman lambs from body measurements with different sample sizes*

n	Model	*Equation*	P	R^2	MSE
10	Linear	BW = -12.516 + 0.811BL	0.040	0.66	17.16
20	Linear	BW = -11.986 + 0.793BL	0.035	0.69	15.44
30	Linear	BW = -9.542 + 0.707BL	0.012	0.81	13.11
50	Linear	BW = -9.233 + 0.765BL	0.003	0.90	11.08

determined in Logistic and the lowest MSE value (14.76) was determined in Saturation growth models. When sample size was kept as 20, the highest R^2 value (0.71) and the lowest MSE value (13.44) was detected in Saturation growth models.

Considering the different sample sizes, the results of the linear regression model are given in *Table 3*. According to these results, the R^2 value was 0.66 and the MSE value was 17.16 in the model with a sample size of 10, and the R^2 value was 0.69 and the MSE value was 15.44 in the model with a sample size of 20. In addition, the R^2 value was 0.81 and the MSE value was 13.11 in the model with a sample size of 30, and the R^2 value was 0.90 and the MSE value was 11.08 in the model with a sample size of 50. According to these obtained values, it was found that as the sample size increased, the R^2 value increased and the MSE value decreased. According to different sample sizes, these coefficients showed that there is more similarity between the linear and nonlinear methods.

Table 4 shows the results of the evaluators of goodness of fit (R^2_{adj}, AIC, BIC, MEP) for each model. Considering the R^2_{adj} value, the highest value was obtained as Incomplete gamma (0.8464), Logistic (0.7921), Allometric (0.7744) and Exponential (0.7744) with sample size 50. The low MEP values, the lowest values were obtained as Incomplete gamma (10.9096), Exponential (11.0864) and Logistic (11.3212), respectively. The lowest R^2_{adj} value was obtained as Allometric (0.3481), Exponential (0.3600), Saturation growth (0.3721) and Incomplete gamma (0.3969) with sample size 10. Considering the high MEP values, the highest values were obtained as Incomplete gamma (24.8530), Exponential (24.2070) and Allometric (20.6392), respectively. Considering the AIC value, the lowest value was obtained as Incomplete gamma (175.3682), Logistic (187.7116), Allometric (188.9002) and Exponential (189.4751) with sample size 50. The lowest BIC values were obtained as Incomplete gamma (182.4006), Exponential (198.6672) and Logistic (202.1524), respectively.

Table 4. *Results of the quality of fit evaluators for the Morkaraman sheeps with different sample sizes*

n	Model	R^2_{adj}	AIC	BIC	MEP
10	Allometric	0.3481	204.9446	219.7146	20.6392
	Logistic	0.4096	198.4871	213.9151	19.7252
	Saturation growth	0.3721	198.4871	213.7989	15.6051
	Exponential	0.3600	210.0728	226.2827	24.2070
	Incomplete gamma	0.3969	204.9446	220.2445	24.8530
20	Allometric	0.4356	198.2946	215.0813	21.6386
	Logistic	0.4225	210.8582	226.7405	20.2056
	Saturation growth	0.5041	205.5648	222.9836	21.3546
	Exponential	0.3600	204.9446	222.7792	17.3273
	Incomplete gamma	0.4624	208.5478	224.3540	15.1510
30	Allometric	0.5476	206.7871	223.7148	16.7722
	Logistic	0.6241	202.1458	218.5526	14.6922
	Saturation growth	0.6084	199.2580	216.1218	15.4842
	Exponential	0.6241	197.6542	218.8890	14.6692
	Incomplete gamma	0.6724	192.8586	214.4148	12.5628
50	Allometric	0.7744	188.9002	202.3128	12.2018
	Logistic	0.7921	187.7116	202.1524	11.3212
	Saturation growth	0.7396	192.1541	216.7005	13.4086
	Exponential	0.7744	189.4751	198.6672	11.0864
	Incomplete gamma	0.8464	175.3682	182.4006	10.9096

Discussion

Topal and Macit [10] were reported that in their study in 66 Morkaraman sheep, as a result of multiple regression analysis, the R^2 value of body length affecting body weight was 0.282 and MSE value was 31.702, respectively. Ibrahim et al.[11] revealed that CG and its combination with other linear body measurements can effectively define the body weight in Batur sheep. However, the highest R^2 of 0.782 was observed when CG and BL were used as predictors. As a result of the different nonlinear models used to estimate the body weight of Morkaraman sheeps, according to the all sample sizes Incomplete gamma model is the most appropriate model when R^2, MSE, R^2_{adj}, AIC and BIC values were taken into account. Rather et al.[12] were emphasized that the coefficient of determination (R^2) is succesful to estimate body weight from body measurements in Kashmir Merino sheeps. Considering the sample sizes of 30 and 50, the highest R^2 values (0.82-0.92) and the lowest MSE values (11.88-11.94) were found in the Incomplete gamma model, respectively. Demir and Sahinler [13] using nonlinear Brody, Bertalanffy, Logistic, Gompertz and Richards models in their study, selected the model with high coefficient of determination (R^2) and low mean square error (MSE) as the best model to describe growth. In conclusion, Richards and Logistic models were the best predictors of overall growth of lambs in nonlinear models. Among the groups, the lowest MSE and the highest R^2 were obtained in the second group and in the Logistic model. According to this model; R^2 and MSE were found to be 0.999±0.0002 and 0.41±0.060, respectively. Macedo-Barragan et al.[2] reported that according to all non-linear models R^2 value is calculated higher than 0.75 for estimating body weight from body measurements.

The nonlinear regression analysis with R^2 calculated for growth in Awassi lambs showed that the relationship derived from the regression weight in weight at 6 months at weaning according to the exponential function was considered to be the best since the R^2 value was 0.69 [14].

Bilgin et al.[15] reported that Brody is the best model for describing as unfit between body weight and age in sheeps. And also, body measurement in farm animals is used to decide the apparent identity and growth pattern [16,17].

Raungprim et al.[18] used three nonlinear regression models: exponential, polynomial quadratic and power models to analyze the relationship between body parameters and body weight. As a result, it was revealed that the power model gave the best HG and body weight relationship model with the highest R^2 (0.9662, 0.9748 and 0.9702) respectively in swamp buffaloes. Topuz [19] calculated the mean membership degree (MDM) and mean square error (MSE) as MDM=0.896 and MSE=4.871, respectively, in order to decide the adequacy of the model by using the fuzzy logic approach-based posibilistic logistic regression

method together with its theoretical background in dairy cattle. According to these values, it was decided that the fit of the model was good.

Considering small sample sizes as 10-20, Logistic and Saturation growth models are more suitable than Allometric, Exponential and Incomplete gamma models for predicting body weight from body length measures. According to the linear model, the highest R^2 and the lowest MSE is obtained from the group of 50 sample size. Considering 30-50 sample sizes group, according the R^2_{adj}, AIC, BIC and MEP values Incomplete model is more appropriate model than the others.

It is concluded that according to the small sample sizes (10-20), Logistic and Saturation growth models can be used to determine the body weight by using body length, on the other hand, Incomplete gamma model is more succesful to estimate body weight when sample size is bigger than 20.

Availabity of Data and Materials

The author declares that data supporting the study findings are also available to the corresponding author.

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Declaration of Conflict of Interest

There was no conflict of interest in regards to author reporting his findings.

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Research Article

Molecular Detection of *Toxoplasma gondii* in Ewes Placenta in Northeastern Algeria

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Abstract: The present study aimed at the direct detection of parasitic DNA in placenta samples of ewes by PCR targeting the B1 gene of *Toxoplasma gondii*. We identified also the possible risk factors associated with the infection. A total of 307 female sheep from 23 farms were collected between 2019 and 2020 in the Tebessa region in northeastern Algeria. Data showed that *T. gondii* DNA was detected in 114 of the 307 tested females (37.1%). The on-farm molecular prevalence was 82.6%. High rates were revealed in Bir Al Ater (32.4%) and Tlidjen (43.6%). The level of contamination was high in farms applying a sedentary mode (47.6%); this system tends to have a positive effect on the prevalence of toxoplasmosis (P<0.05; 95% CI: 0.409-0.544). Moreover, it appears that the relationship between the presence of cats on the farm and the prevalence of the disease was significant (P<0.05; 95% CI: 0.445-0.597). In the same context, primiparous females were 2.54 times more likely to be infected with *T. gondii* than multiparous animals (P=0.001). To conclude, the prevalence noticed in the present study indicates a generalized exposure of sheep to *T. gondii* in the northeast of Algeria, which represents a major risk for animal and public health. Therefore, management measures should be implemented and improved in the farms of this region for better disease control and eradication.

Keywords: *Algeria, Ewes, Molecular detection, Placenta, Toxoplasma gondii*

Cezayir'in Kuzeydoğusundaki Koyun Plasentalarında *Toxoplasma gondii*'nin Moleküler Tespiti

Öz: Bu çalışmada, *Toxoplasma gondii*'nin B1 genini hedef alan direkt PCR ile koyun plasenta örneklerinde parazitik DNA'nın saptanması amaçlandı. Enfeksiyona ilişkin olası risk faktörleri de değerlendirildi. Cezayir'in kuzeydoğusundaki Tebessa bölgesinden 2019-2020 yılları arasında 23 çiftlikten toplam 307 dişi koyun örneklendi. Veriler, test edilen 307 dişi koyunun 114 (%37.1)'ünde *T. gondii* DNA'sının tespit edildiğini gösterdi. Çiftlikteki moleküler prevalans %82.6 olarak saptandı. Bir Al Ater (%32.4) ve Tlidjen'de (%43.6) pozitiflik yüksek orandaydı. Yerleşik mod uygulayan çiftliklerde kontaminasyon seviyesi yüksekti (%47.6); bu sistem, toksoplazmoz prevalansı üzerinde olumlu bir etkiye sahip olma eğilimi gösterdi (P<0.05; %95 CI: 0.409-0.544). Ayrıca, çiftlikte kedi varlığı ile hastalığın prevalansı arasında anlamlı bir ilişki saptandı (P<0.05; %95 CI: 0.445-0.597). Aynı bağlamda, primipar dişilerin, multiparlara göre *T. gondii* ile enfekte olma olasılığı 2.54 kat daha fazlaydı (P=0.001). Sonuç olarak, bu çalışmada saptanan prevalans, Cezayir'in kuzeydoğusundaki koyunların *T. gondii*'ye genel olarak maruz kaldığını göstermektedir ve bu durum, hayvan ve halk sağlığı için büyük bir risk teşkil etmektedir. Bu nedenle, hastalık kontrolü ve eradikasyonunun daha iyi yapılabilmesi için bu bölgedeki çiftlik yönetimine ait uygulamaların iyileştirilmesi ve uygulanması gerekmektedir.

Anahtar sözcükler: *Cezayir, Koyun, Moleküler tespit, Plasenta, Toxoplasma gondii*

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Introduction

Toxoplasma gondii is an important protozoan parasite found worldwide that potentially infects all warm-blooded vertebrates, including mammals, birds, and humans [1]. *T. gondii* cysts have been found in the tissues of pigs, sheep, goats, and other animals. They are transmitted to humans by the accidental ingestion of oocysts located in cat's feces or by eating raw or undercooked meat containing cysts [2]. *T. gondii* is the primary parasite responsible for both sheep and goat abortion [3]. These species represent a significant source of infection, mainly in those regions or countries where sheep and goat meat is routinely consumed [1]. As this parasite affects both animals and humans, food-borne transmission is one of the major sources of *T. gondii* infection by ingesting tissue cysts from under-cooked meat [4].

Toxoplasmosis is relatively common in small ruminants [5]. Although most infections in these species are asymptomatic, there can be abortions, fetal mummification, stillbirths, and birth of weak lambs and kids [6]. The exposure of a pregnant ewe to primary infection with *T. gondii* may, depending on its stage of gestation when the infection occurs and the infective dose, result in the death of the fetus and lead to significant losses in affected flocks as a result of barrenness, abortion, and stillbirths. The birth of clinically normal, infected lambs usually results from a primary infection contracted during the latter part of pregnancy [7]. Once infected, ewes develop good protective immunity against the parasite, which protects against disease in subsequent pregnancies [8].

Infectious abortion is one of the major flock health problems faced by sheep farmers and has a significant financial impact on production. Toxoplasmosis is one of the main causes of infectious reproductive failure in small ruminants in the world [8,9].

Small ruminant production is one of the most important sources of meat in Algeria and plays a vital role in the country's food security [10]. However, the prevalence of *T. gondii* infection in these animals in Algeria remains largely unknown. Only a few data on the infection of small ruminants are available, not updated or just limited to small areas. However, some epidemiological studies as part of final theses and doctorates in medical sciences have provided an idea of seroprevalence in sheep that varied from 8.28% to 35.37% [11,12]. Only one molecular study of blood samples was conducted with a recorded prevalence of 35.2% in ewes [13].

Therefore, this current work aimed to report the results of a cross-sectional survey on the molecular prevalence of *T. gondii* infection in placenta samples of ewes in the northeastern part of Algeria, where these animals have great economic importance. The study also assessed the possible risk factors associated with the infection to estimate the risk of toxoplasma abortion in this region.

Material and Methods

Ethical Statement

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

Period and Type of Study

Our work covered the farrowing and abortion seasons from September to January and March to May for one year. This is a descriptive exploratory study conducted from September 2019 to October 2020.

Study Sites and Materials

The study took place in the steppe of Tebessa in the northeastern of Algeria (35°24'15.0"N 8°07' 27.0" E), which constitutes the predilection area of sheep and goat breeding. This zone is mountainous and is 960 m above sea level. It has a semi-arid climate characterized by hot summers and cold, wet winters with a rainfall averaging 363 mm per year. The study area can be broadly divided into three departments of the central zone of Tebessa, including Bir Al Ater, Tlidjen, and Negrine *(Fig. 1)*.

The work was conducted on ewes that had abortion problems within 8 days. Flock selection was based on abortion rates exceeding 5% since a lower rate is considered normal in a flock and does not attract the attention of the farmer [14]. A total of 307 female sheep from 23 farms were collected between September 2019 and October 2020. Eight farms were located in Bir Al Ater, 10 in the Tlidjenarea, and 5 in Negrine.

Data Collection

In each case of abortion, the commemoratives were noticed for each animal and each farm. Data were collected on age and the reported stage of pregnancy at the time of the abortion. In addition, herd-level information was collected (breeding system, abortion history, and presence of stray cats in the farm) and used as explanatory variables.

Samples Collection

In the selected farms, samples concerned only females with abortion. Placentas were collected under sterile conditions using a disposable scalpel blade. One cotyledon from each placenta was collected and stored in sterile 1.5 mL tubes at -20°C until analysis in the laboratory.

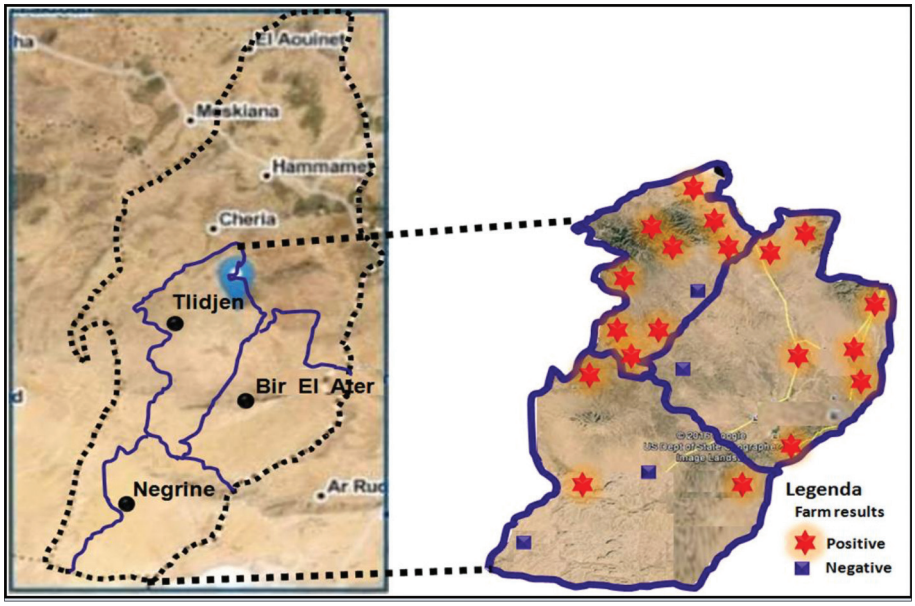

Fig 1. Location map of sampled farms in the three study areas of Tebessa in Northeastern Algeria (Bir Al Ater, Tlidjen, and Negrine). The site is located by a box

DNA Extraction and Nested PCR for the Detection of *T. gondii*

Samples were partially thawed at laboratory temperature for 10 min. DNA was extracted from 100 mg of homogenized cotyledon using a commercial QIAmp DNA tissue Mini Kit (Qiagen, France) according to the manufacturer's protocol. Due to the high concentration of DNA, the purified DNA samples were resuspended in ultrapure water. DNA concentrations were determined by spectrophotometric analysis, and all samples were diluted to a final concentration of 300 ng/µL and stored at -20°C before PCR analysis.

The presence of *T. gondii* was detected by conventional PCR using the nucleotide sequence of the B1 gene as a target. A pair of primers, JW63: (5'-GCACCTTTC GGACCTCAACCG-3') and JW62 (5'-TTCTCGCCTCA TTTCTGGGTCTAC-3') were used to amplify a 286 bp fragment of the gene target as described by Pelloux et al.[15]. The PCR reaction was performed in 50 µL of a mixture containing 5 µL of sample DNA diluted with 17.5 µL of water of injections, 1.25 µL of each primer, and 25 µL of Master Mix. The latter is a prepared solution containing: 1x Taq polymerase buffer supplemented with $MgCl_2$ (3 µM), 1.6 µM of each dNTP, and 50 Units/mL Taq DNA polymerase (GoTaq®, Promega). Amplification was performed on a thermal cycler (Applied Biosystem 2700) by incubation in 4 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and final 5 min at 72°C. As a positive control, *T. gondii tachyzoites* (BALBcstrain) were obtained from ascites of previously infected mice. Negative controls (double distileed water) were included in each set of PCR reactions. Amplified products were analyzed by 2% agarose gel electrophoresis and visualized on a UV screen by ethidium bromide staining.

Statistical Analysis

A herd was considered positive if at least one animal in the herd was tested positive. The prevalence was calculated by dividing the number of positives by the number of tested animals. The p-value and the confidence interval of 95% (95% CI) were also calculated. The analysis was conducted globally (at the level of the herd) and then individually. Pearson's Chi-square was used to test the different variables. All questionnaire responses were included in the statistical analysis as independent variables.

Statistical analyses were finalized via logistic regression using XLSTAT software version 2016.02.28451. First, univariate logistic regression analysis was performed for all hypothesized risk factors to investigate possible relationships between *T. gondii* prevalence and the different risk factors. Second, all variables were entered into a multivariate model, developed by backward elimination until all remaining variables were significant (P≤0.05).

Results

T. gondii DNA was found in 19 (82.6%; 95% CI: 90-100%) of the 23 farms. At the individual level, *T. gondii* was detected in 114/307 placenta samples indicating an overall molecular prevalence of 37.1% *(Table 1)*. The spatial distribution of positive farms is shown in *(Fig. 1)*. Intra-herd prevalence for infected farms ranged from 18.2% to 61.9%. Negative animals were found on all farms. The infection rate was more than 50% in 17.4% of the herds (4 herds), and in 14 farms (60.9%), more than 25% or less than 45% of animals were positive. Only 1 farm had a percentage below 20% *(Fig. 2)*.

The results obtained from the univariate analysis of the risk factors are presented in *Table 2*. The molecular prevalence

Table 1. *Toxoplasma gondii infection rates and confidence interval (CI) at 95% in the studied population*

Animals			Herds		
Number of Tested Animals	**Number of Positive Animals (n) (%)**	**95%CI**	**Number of Tested Herds**	**Number of Positive Herds (n) (%)**	**95%CI**
307	114 (37.1%)	[31.72-42.53]	23	19 (82.6%)	[67.11-98.09]

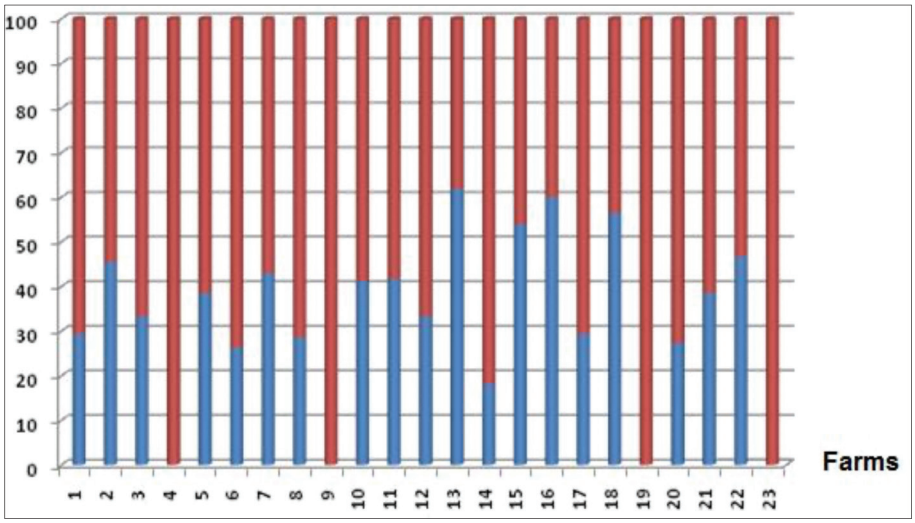

Fig 2. The proportion of *T. gondii*-positive *(blue)* and negative *(red)* animals in the 23 sheep farms

Table 2. *Descriptive statistics and univariate analysis of the effect of different variables on toxoplasmosis prevalence*

Variable	Category	Number of Tested Animals	Number of Positive Animals	%	95% CI	P-Value
Collection area	Bir Al Ater	105	34	32.4	0.234- 0.413	[1] VS [2] Chi2 = 3.25 P=0.07
	Tlidjen	149	65	43.6	0.357-0.516	[1] VS [3] Chi2 =0.27 P=0.6
	Negrine	53	15	28.3	0.162-0.404	[2] VS [3] Chi2 =3.76 P=0.05
Age	Primiparous	137	77	56.2	0.479-0.645	Chi2 = 36.41 P<0.05
	Multiparous	170	37	21.8	0.156-0.280	
Abortion stage	Early gestation (1-90 days)	139	61	43.9	0.356-0.521	Chi2 = 4.92 P<0.05
	End of gestation (90-145 days)	168	53	31.5	0.245-0.386	
Breeding mode	Sedentary	210	100	47.6	0.409-0.544	Chi2 = 27.66 P<0.05
	Transhumant	97	14	14.4	0.074-0.214	
History of abortions	Yes	236	99	41.9	0.357-0.482	Chi2 = 9.66 P<0.05
	No	71	15	21.1	0.116-0.306	
Presence of cats	Yes	167	87	52.1	0.445-0.597	Chi2 = 32.86 P<0.05
	No	140	27	19.3	0.128-0.258	

Statistically significant variables are indicated by bold typing; [1] Bir Al Ater, [2] Tlidjen, [3] Negrine

in both sites; Bir Al Ater (32.4%) and Tlidjen (43.6%) was higher. This shows that ewes from the Negrine area influence the prevalence of *T. gondii* (P=0.05; 95% CI: 0.162- 0.404). Regarding age, the highest prevalence was recorded in primiparous females with 56.2%, compared to multiparous females (21.8%). It appears that there is a significant difference (P<0.05; 95% CI: 0.156- 0.280) in Toxoplasma prevalence between the two age groups. Besides, statistically significant results were revealed according to the gestational stage (P<0.05; 95% CI: 0.356-0.521). Regarding the variable "rearing mode", the level of contamination was high in farms applying a sedentary

Table 3. Multivariate logistic regression analysis data of the supposed risk factors in relation to toxoplasmosis

Variable	Category	Odds Ratio	95% CI	P-Value
Age	Primiparous	2.540	1.436-4.495	0.001
Abortion stage	Early gestation (1-90 days)	1.155	0.641-2.081	0.631
Breeding mode	Sedentary	3.972	2.038-7.742	< 0.0001
Presence of cats	Yes	3.188	1.725-5.892	0.0001

Statistically significant variables are indicated by bold typing

mode (47.6%); this system tends to have a positive effect on the prevalence of toxoplasmosis (P<0.05; 95% CI: 0.409-0.544). Concerning the risk factor "previous abortion"; in fact, the prevalence of toxoplasmosis increased with the existence of a history of abortion (P<0.05; 95% CI: 0.357-0.482). Moreover, it appears that the relationship between the presence of cats on the farm and the prevalence of the disease is significant (P<0.05; 95% CI: 0.445-0.597).

Four risk factors for toxoplasmosis were retained in the multivariable model, with three variables significantly associated with a higher probability of herds being positive *(Table 3)*. For the farm management variables, the risk of toxoplasmosis positive was 3.97 times higher in sedentary livestock (P<0.0001). Furthermore, the presence of a cat on the farm was significantly associated with a high rate of toxoplasmosis (OR=3.18, P=0.0001). Regarding the abortive syndrome, only age was identified as a potential risk factor for *T. gondii* infection. In this study, primiparous animals were 2.54 times more likely to be infected with *T. gondii* than multiparous animals (P=0.001). In contrast, the variable stage of gestation was not significantly related to the risk of developing the disease (OR = 1.15, P=0.63).

Discussion

Toxoplasma gondii is a food- and waterborne parasite that infects nearly all mammals, including humans [16,17]. Sheep and goats are important sources of infection for humans, representing an important public health role. In addition, toxoplasmosis is an important cause of neonatal mortality in sheep and goats, resulting in reproductive and economic losses worldwide [18,19]. Epidemiological data on *T. gondii* infections in animals for human consumption are not regularly collected and the current lack of standardization of diagnostic techniques and protocols should be taken into account when comparing seroprevalence data [20]. Molecular analysis of *T. gondii,* which detects the circulating parasites would be useful for the final diagnosis. Serological findings are only an indication of infection, while molecular detection of *T. gondii* in blood or other samples provides the presence of the parasite in the body [21,22].

Previously in Algeria, the prevalence of *T. gondii* infection in animals was assessed by serological tests [17]. In our present work, the detection of *T. gondii* in ewes placenta was carried out by molecular analysis.

Data revealed a herd prevalence of 82.6%. This rate is among the previously reported values in Algeria, obtained from the study of toxoplasmosis seroprevalence in sheep ranged from 57.89%- 100% [23,24]. Other works conducted in European countries have shown variable seroprevalence: 98.4% and 87.5% of sheep flocks were positive in surveys conducted in Southern Spain in 2020 [25], and in Northern Italy in 2015 [1], respectively.

In the present study, *T. gondii* DNA was detected in 114 females out of 307 placenta samples (37.1%) by conventional PCR based on B1 gene amplification. In a previous study of blood samples, similar values were reported in sheep from the same region (35.2%) [13]. Our results were higher than that of a molecular report confirming the presence of *T. gondii* in 1.69% of sheep carcasses in Northern India [26] and that found by Prasad Sah et al.[27] in Bangladesh (15.52%) in sheep tissue samples. Other previous studies conducted on placental tissue samples have shown different rates. Indeed our result was higher than that found in ewes in Italy (3.5%) [28]. It is also consistent with other data found by PCR in ovine abortion products (fetuses and placenta) in the same country (31.5%) [29].

The divergence in toxoplasmosis prevalence may be explained at first glance by the differences in methodology, in sample size and sampling techniques [30], and in climatic variations and feline density [31,32].

In our study, the "site" effect was associated with the prevalence of *T. godii* in the univariate analysis. Females collected in the regions of Bir Al Ater and Tlidjen showed a high molecular prevalence. A statistical relationship was found between the prevalence of infection and females from the Negrine area, characterized by a high presence of cats. *T. gondii* oocysts excreted by cats remain infective for years under favorable conditions (adequate humidity and temperature) [33].

In tested females, molecular prevalence was positively correlated with age, as reported in previous studies [34,35]. The higher risk of *T. gondii* infection in primiparous ewes suggests that once infected and aborted females generally

do not abort upon re-exposure to the parasite, even though the parasite survives as a cyst until the end of the mother's life. The ewe then harbors bradyzoites and becomes immune after the first infection [36,37].

In the present study, although stage of gestation was not indicated as a risk factor associated with *T. gondii* infection in the multivariate analysis, it was positively associated with the prevalence of toxoplasmosis in the univariate analysis. Females with early abortions had higher prevalence. This variable was also identified as a risk factor in a previous study which is consistent with our finding [9]. In another study performed in Brazil, Silva Filho et al.[38] reported that all abortions occurred in the last months of gestation. Therefore, it has been mentioned that the immune system of the ovine fetus can respond to *T. gondii* at 60 days of gestation or shortly thereafter. Thus, infection before 40 days of gestation is probably due to local suppression of immune mechanisms in the maternal placenta and the immaturity of the fetal immune system. In contrast, infection between 40 and 120 days may be attributed to insufficient immunity to confer protection until the last month before birth [39].

Consistent with our results on husbandry, the multivariate model analysis detected two risk factors for toxoplasmosis. Indeed, extensive management was certainly an important risk factor associated with infection. The high rate was observed in farms with sedentary breeding. This result is in agreement with that of Heidari et al.[32] who reported that sedentary managed animals are much more able to acquire Toxoplasma. In a completely enclosed farm, the infection risk is almost limited to the introduction of new animals and the presence of vectors such as rodents or insects. In contrast, in extensive farming, where animals are potentially in contact with animals from other farms, wildlife, or a contaminated environment, the infection risk is much higher [40]. Also, the presence of felines on farms increases the risk of toxoplasmosis. These findings are in agreement with the biology of *T. gondii* and emphasize that the presence of cats plays a central role in *T. gondii* infection. This may indicate high contamination of production areas (both pastures and containment facilities) with *T. gondii* oocysts, thus necessitating sanitary control and prevention measures on farms. The significant correlation with the prevalence of toxoplasmosis has been cited several times [41]. Finally, and consistent with our previous observations, the variable "history of abortions" was identified as a risk factor for the disease in the univariate analysis. Molecular prevalence was positively associated with the existence of a history of abortions in the farms, which was previously revealed by Gharekhani [31]. Therefore, the infection is likely to reappear after a few years due to the increased number of susceptible animals in the replacement generations [13].

Toxoplasma gondii infection has occurred with a high molecular prevalence in ewes. The results of this study show that toxoplasmosis is present in the sheep population of Tebessa, confirming that this species could be an important source of *T. gondii* among consumers in this area. Hygiene and dietary advice should be disseminated to consumers of sheep meat and particularly to vulnerable individuals (pregnant women and immunocompromised patients not yet immunized for this disease), including the importance of consuming sufficiently cooked meat. The data obtained further underestimates the risk factors associated with *T. gondii* infection and the relationship between the parasite and these small ruminant hosts. Such information may be useful for veterinarians and farmers to develop or improve toxoplasmosis control plans in herds in the study areas and in areas where farming systems are similar to those described in the current work. In addition, given that Algeria raises sheep for domestic consumption, isolation of *T. gondii* from sheep with molecular characterization of isolates will be necessary to assess better and understand the risk of ovine toxoplasmosis to human health. If isolates of virulent genotypes are found, this will increase the risk of potentially serious infestation to humans.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (N. Ait Issad) on reasonable request.

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Competing Interests

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

Author Contribution

NAI: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing, Review and Editing. KA, SB, RB, HHA, TGAH, NM: Resources, Methodology, Validation, Formal Analysis, Investigation, Project administration. DD: Statistical analysis. RK: Investigation, Writing - Review & Editing, Project administration. DK: Supervision, Investigati on, Writing - Review & Editing, Project administration.

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Research Article

Effect of Different Litter Size on The Rate of Postpartum Uterine Involution in Hu Sheep

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Abstract: In this study, we investigated the effects of different litter sizes on the rate of uterine involution in Hu sheep. Using B-mode ultrasonography, we recorded changes in the uterine horn diameter and the maximum uterine caruncle diameter in 60 primiparous Hu sheep from days 0 to 45 postpartum. The uterine horn diameter decreased gradually postpartum from 79±1.42 mm at day 0 to 10.87±0.5 mm at day 45 with singleton parturition, from 91±6.58 mm at day 0 to 10.63±0.32 mm at day 45 with twin parturition, and from 107±3.67 mm at day 0 to 11±0.87 mm at day 45 with triplet parturition. The time to complete uterine involution postpartum was 30, 35 and 40 days postpartum in singleton, twin and triplet parturitions, respectively. (P<0.05). The maximum uterine caruncle diameter also decreased gradually from 12.43±0.91 mm at day 3 to 8.82±0.27 mm at day 10 with singleton parturition, from 12.14±1.19 mm at day 3 to 8.31±0.94 mm at day 10 with twin parturition, and from 12.5±0.66 mm at day 3 to 7.94±0.95 mm at day 10 with triplet parturition (P>0.05). Thus, different litter sizes had a greater effect on postpartum uterine horn recovery than on the uterine caruncle. Furthermore, the rate of uterine involution in ewes with singleton parturition was significantly higher than that with triplet parturition. Our findings provide a reference for improving the reproductive performance of Hu sheep.

Keywords: *Hu sheep, Litter size, Uterine involution, Ultrasound*

Hu Koyunlarında Farklı Batın Genişliklerinin Doğum Sonrası Uterus İnvolüsyonu Oranına Etkisi

Öz: Bu çalışmada, Hu koyunlarında farklı batın genişliklerinin uterus involüsyon hızına etkilerini araştırdık. B-mod ultrasonografi kullanarak, doğum sonrası 0 ila 45. gün arasındaki 60 primipar Hu koyununun uterus kornu çapı ve maksimum uterus karunkül çapındaki değişiklikleri kaydettik. Doğum sonrası 0 ile 45. gün arasında uterus korn çapları tekil doğum yapanlarda 79±1.42 mm'den 10.87±0.5 mm'ye, ikiz doğuranlarda 91±6.58 mm'den 10.63±0.32 mm'ye ve üçüz doğuranlarda 107±3.67 mm'den 11±0.87 mm'ye düştü. Doğum sonrası uterus involüsyonunun tamamlama süresi, tekil, ikiz ve üçüz doğumlarda sırasıyla 30, 35 ve 40 gündü (P<0.05). 3. günden 10. güne maksimum uterus karunkül çapları tekil doğumlarda 12.43±0.91 mm'den 8.82±0.27 mm'ye, ikiz doğumlarda 12.14±1.19 mm'den 8.31±0.94 mm'ye ve üçüz doğumlarda 12.5±0.66 mm'den 7.94±0.95 mm'ye düştü (P>0.05). Bu nedenle, farklı batın genişliklerinin, doğum sonrası uterus kornularının iyileşmesi üzerine etkisi, rahim kıkırdağına göre daha büyüktü. Ayrıca, tekil doğum yapan koyunlarda uterus involüsyon oranı, üçüz doğum yapana göre önemli ölçüde daha yüksekti. Bulgularımız, Hu koyunlarının üreme performansının iyileştirilmesi için bir referans sağlamaktadır.

Anahtar sözcükler: *Hu koyunu, Batın genişliği, Uterus involüsyonu, Ultrason*

Introduction

The reproductive performance of small ruminants has important economic benefits, as it determines the annual number of offspring. Uterine involution refers to a physiological process in which the uterus of a dam returns to its non-pregnant state and function after parturition. Several techniques are used to study uterine involution, such as dissection, hormone content determination, and ultrasonography [1-3]; however, ultrasonography is a non-invasive technique that is not only less harmful to dams, but also allows intuitive and accurate monitoring

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of uterine changes during uterine involution. Uterine involution has also been extensively studied in other animals, including Holstein cows [4-6], horses [7], in pigs [8-10], in bitches [11] and cats [12].

Uterine involution in sheep is affected by many factors including nutrition level, season of parturition, breed and parity [13-17]. The time of uterine involution in subtropical sheep lambing in February was 29.4±1.2 day, lambing in June was 33.9±1.1 days [14]. Medan et al.[15] showed that the time to complete uterine involution in Libyan goats was 28-35 days, while Hauser et al.[16] reported that the process was completed in approximately 17 days in an improved German land breed of sheep. The uterine body and caruncle of Baladi goats was basically recovered by day 19 postpartum, and this time was not related to parity [17]. In this study, we investigated the effect of litter size on the rate of uterine involution in primiparous Chinese Hu sheep using B-mode ultrasonography. This breed can produce up to three litters of 1-7 lambs within two years and has excellent traits such as strong environmental adaptability, perennial oestrus, good lactation performance, and rapid growth and development. Adult rams and ewes weigh approximately 65 kg and 40 kg, respectively, and are ready for mating at 6 months of age [18].

This investigation of the effects of different litter sizes on the rate of uterine involution in Hu sheep will provide a reference for improving the reproductive performance of Hu sheep.

Material and Methods

Experimental Animals

A total of 300 Hu sheep aged between 8 and 18 months and weighing 40-60 kg, were included in this study from May to June 2021. All sheep were from the breeding base of Runkangyuan, Xinjiang, China, and housed under conditions of natural light. Ewes were fed a complete diet prepared according to their nutritional requirements and allowed free access to water with mineral salts provided in a licking block throughout the experiment. All lambs were suckled by the ewes.

Primiparous Hu sheep were artificially inseminated after estrus synchronization treatment. Then they were submitted to an ultrasonographic evaluation to determine the number and location (on the same side of the uterine horn) of fetuses was determined by B-mode ultrasonography on day 40 of the pregnancy.

Experimental Design

According to the number of lambs, 60 ewes with a mean body condition score (BCS) of 3.48/5 according to Russel et al.[19] in the northern hemisphere were selected for analysis of uterine involution and allocated to the following groups: Group A, singleton parturition (n=20; average weight = 41.2 kg); Group B, twin parturition (n=20; average weight = 40.3 kg); and Group C, triplet parturition (n=20; average weight = 40.8 kg). Postpartum uterine involution was monitored in all sheep by transrectal ultrasonography measurement of the uterine horn diameter and the maximum uterine caruncle diameter on days 0, 3, 7, 10, 14, 18, 22, 26, 30, 35, 40 and 45.

Ultrasonography and Image Analysis of the Uterine Horn and Caruncle

Uterine involution was monitored in all postpartum sheep in the standing position by transrectal ultrasonography (7.5-MHz linear-array; HS-1600, HONDA, Japan) performed by an operator wearing sterile gloves. After application of a layer of coupling agent, the probe was slowly inserted into the cleaned rectum with the scan detection window facing down. The bladder was used as a reference object to rotate the left and right sides for scanning to obtain the best field of view of the uterine horn and caruncle [20,21]. Transrectal ultrasonography was performed at the uterine angular curvature. Images of the section corresponding to the maximum uterine caruncle diameter were recorded, and the length of the external diameter was measured. The diameter of uterine caruncle was determined by measuring at least three uterine chambers [22]. The section diameter was calculated as (long axis length + short axis length)/2.

Statistical Analysis

Data were statistically analyzed using SPSS 20.0 (Statistical Product and Service Solutions 20.0) software. All data were expressed as mean ± standard error of mean (SEM). One-way ANOVA was used to evaluate differences between groups. Duncan's test was used to evaluate differences in the uterine horn diameter and maximum uterine caruncle diameter. $P<0.05$ was set as the threshold for statistical significance.

Results

Ultrasonography of the Uterine Horn of Hu Sheep in the Postpartum Period

Images of the bladder filled with urine *(Fig. 1-A2)* and when empty *(Fig. 1-B5)* were used as a reference for ultrasonography of the uterus. The uterine horns were identified by transrectal ultrasonography using the bladder as a reference. The size and volume of the uterine horns were gradually reduced with time postpartum in all groups *(Fig. 1)*.

Although the uterine horn was too large to be displayed by ultrasonography before day 3 postpartum, uterine residual fluid was clearly observed *(Fig. 1-A1 white circle)*. The structures of the uterine wall, including the mucosal layer,

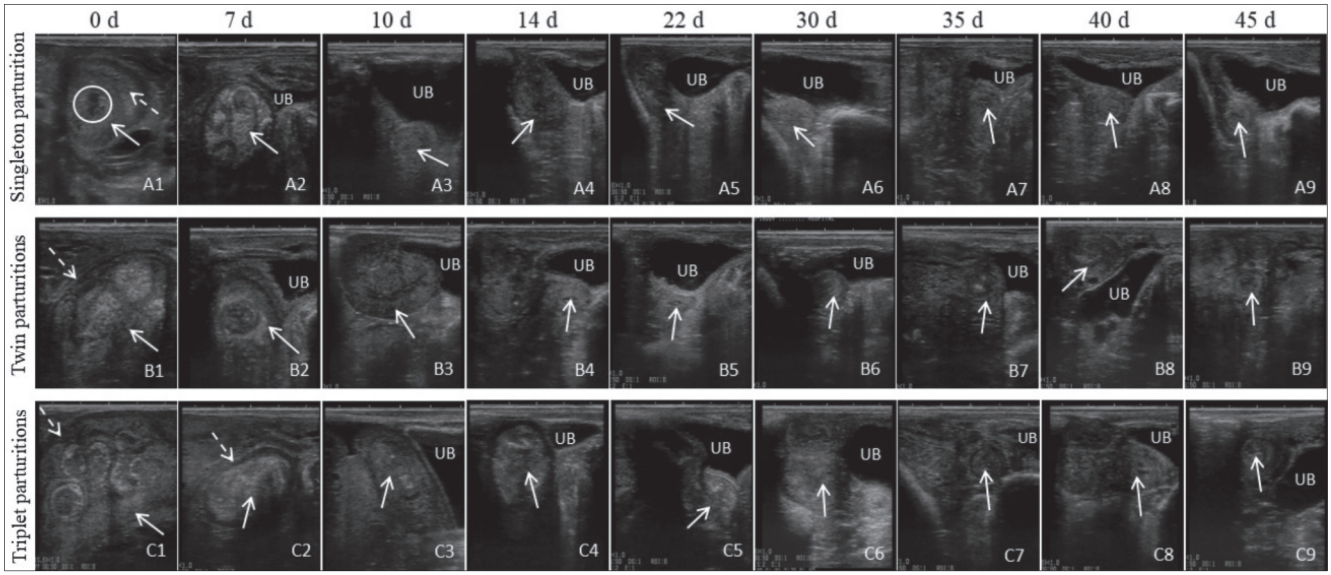

Fig 1. Ultrasonography of uterine horns in Hu sheep 0-45 days postpartum, as detected with a 7.5-MHz transducer. UB, bladder; uterine horn *(solid arrow);* uterine wall *(dashed arrow);* uterine residual fluid *(white circle).* The number of days postpartum are shown above the images

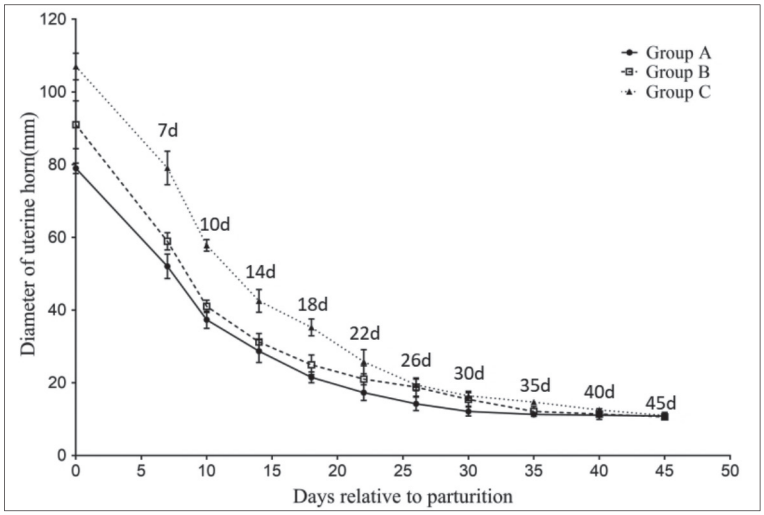

Fig 2. Changes of uterine horn diameter 0-45 days postpartum in Hu Sheep

were also clearly visible *(Fig. 1-B1, dotted arrow)*. Until day 7 postpartum, the whole uterine horn was visible in cross-section. The image of the uterine wall structure blurred gradually from day 10 postpartum and the uterine residual fluid disappeared *(Fig. 1-A3, solid arrow)*. The uterine horn contracted gradually from day 14 to day 26 postpartum. In Groups A, B and C, uterine involution was completed on days 30, 35 and 40 postpartum, respectively. On day 45 postpartum, the uterine horns were similar in size and shape (oval or round) in all groups *(Fig. 1-A9,B9,C9)*.

Changes in Uterine Horn Diameter of Hu Sheep in the Postpartum Period

The diameter of the uterine horn decreased rapidly from day 0 to day 14 postpartum, and was followed by a slow recovery. On day 7 postpartum, the diameters of the uterine horn in Groups A, B and C were 52±0.75 mm, 58.9±2.39 mm and 63.3±13.2 mm, respectively. However, by day 14 postpartum, the diameters of the uterine horn were 28.7±3.13 mm, 31.2±2.36 mm and 42.5±3.12 mm, representing reductions of 73.86%, 72, 68%, and 67.19% in Groups A, B and C, respectively. The diameters of the uterine horn in Groups A, B and C were stable by days 30, 35 and 40 postpartum, respectively, with uterine horn diameters ≤130 mm ($P<0.05$) *(Fig. 2)*.

Ultrasonography of the Uterine Caruncle of Hu Sheep in the Postpartum Period

The uterine caruncle of sheep is attached to the endometrium, although the shape varies due to differences in the detection positions. The diameter of the uterine caruncle decreases with proximity to the fallopian tube and cervix. Therefore, in this study, the maximum uterine caruncle diameter was measured by B-mode ultrasound detection of a discoid shape at the uterine angular

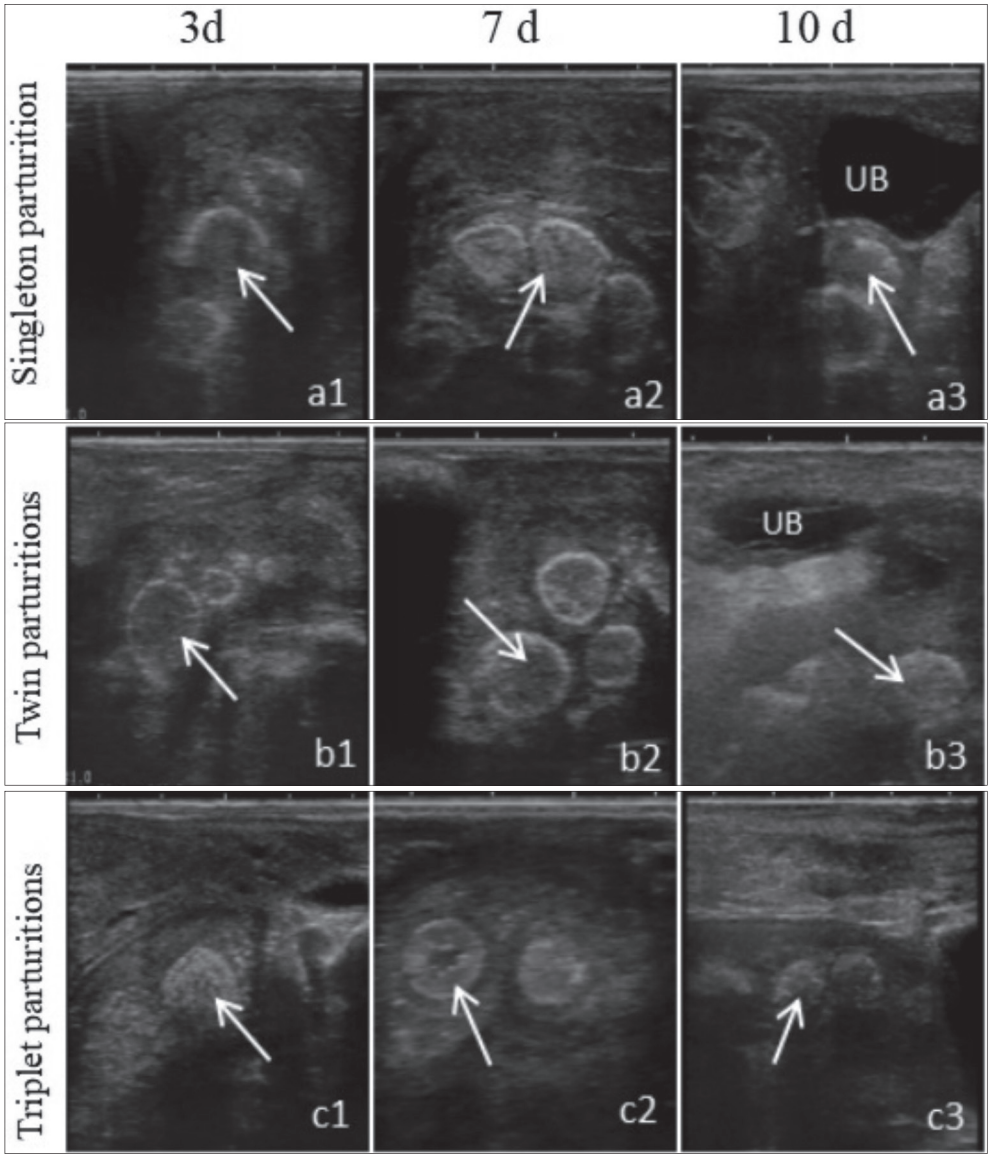

Fig 3. Ultrasonography of uterine caruncle in Hu sheep 3-10 days postpartum, detected with a 7.5-MHz transducer. UB, bladder; uterine caruncle *(solid arrow)*. The number of days postpartum are shown above the images

curvature [23,24]. The contour of the caruncle was clearly seen to be round or oval from days 3 to 10 postpartum and the diameter of the caruncle began to decrease with increasing time postpartum until it was essentially undetectable after day 10 *(Fig. 3)*.

Changes in the Maximum Uterine Caruncle Diameter of Hu Sheep in the Postpartum Period

On day 3 postpartum, the maximum diameters of the uterine caruncle of ewes in Groups A, B and C were 12.43±0.91mm, 12.14±1.19 mm and 12.5±0.66 mm, respectively. By day 7 postpartum, the maximum diameter of the uterine caruncles in Groups A, B and C were reduced to 10.86±0.86 mm, 10.0±0.12 mm and 9.97±1.48 mm, respectively, and 8.82±0.27 mm, 8.31±0.94 mm and 7.94±0.95 mm, respectively, by day 10 postpartum. After day 10, the uterine caruncles in all groups were almost undetectable *(Fig. 4)*.

Discussion

Ultrasonography is widely used in sheep production, not only for disease diagnosis, but also for determination of the fetus number in early pregnancy. B ultrasound has emerged as a non-invasive method for monitoring of uterine involution in sheep. Complete uterine involution is defined as a uterine horn ≤200 mm in transverse diameter and no fluid collection in the uterine cavity [25].

The time to complete uterine involution is affected by different factors. In our study, the process occurred most intensively during the first 14 days postpartum, corresponding to a total reduction of 50%. Thereafter, the

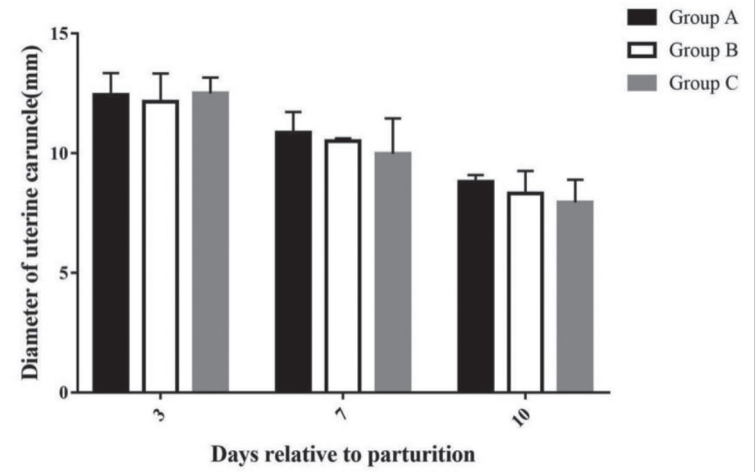

Fig 4. Changes in the maximum uterine caruncle diameter 3-10 days postpartum in Hu sheep

involution rate decreased and stabilization was reached by days 30, 35 and 40 for singleton, twin and triplet parturitions, respectively. Completion of uterine involution has been reported to occur by day 18 postpartum in sheep and by day 21 in goats [26]. Compared with the results of our study, this discrepancy in the time to complete uterine involution may be due to breed differences. Medan et al.[15] showed that uterine involution was completed on day 35 postpartum in ewes after lambing in January and February, and on day 28 postpartum after lambing in March. Hayder et al.[14] also found that the mean interval for complete uterine involution was shorter in ewes after lambing in February, compared to those lambing in June (29.4±1.2 days vs. 33.9±1.1 days). This was similar to the rate of uterine involution after singleton and twin parturitions observed in our study. Gomes et al.[27] found that uterine depth regression stabilized on average on days 35 and 49 postpartum for singleton and twin parturitions, respectively.

Necropsy samples showed revealed the appearance of edema in the degenerated uterine caruncle on day 14 postpartum. Following complete necrosis of the caruncle surface on day 16 postpartum, the surface regained cleanliness, luminescence, although some necrotic caruncle tissue remained attached to the uterine wall at day 21 [28]. Finally, epithelialization of the uterine caruncle completed the regeneration by 28 days postpartum [29]. Ahmed et al.[30] observed uterine caruncles within 10 days postpartum in ewes, which was consistent with the present study. The diameter of the uterine caruncle measured 2.02±0.16 cm on day 2 postpartum and regressed to 1.24±0.17 cm on day 8 [16]. Ioannidi et al.[13] reported that the caruncle was undetectable after day 20 postpartum.

The uterine caruncle and uterine fluid decreased gradually in the first week postpartum. Ababneh and Degefa [17] found the diameter of the uterine caruncle was 2.86±0.43 cm on day 2 postpartum and 1.33±0.22 cm on day 9. In the present study, the uterine caruncle tended to decrease significantly in size in all sheep during the first week postpartum. The diameter of uterine caruncle on day 10 postpartum was significantly smaller than that on day 3 postpartum (8.36±0.78 mm vs. 12.36±0.83 mm; $P<0.05$). Furthermore, there were no significant differences in the diameters of the uterine caruncle among the three groups at 3 days postpartum ($P>0.05$).

In this study, we observed a rapid reduction in the uterine horn diameter from days 7-22 postpartum, followed by a more gradual decreased from days 22-40 postpartum. The time to completion of uterine involution increased significantly postpartum with the number of parturitions ($P<0.05$). Uterine involution was completed by day 30 postpartum in ewes with singleton parturition, by day 35 postpartum with twin parturitions, and by day 40 postpartum with triplet parturitions. Thus, our findings provide evidence that different litter sizes have influence the rate of uterine involution and represent a reference for improving the reproductive performance of Hu sheep.

Availability of Data Materials

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

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contribution

WB Zeng and YP Wang designed the study, conducted the experiments, analyzed the data, and drafted the manuscript. ZL Liu designed the study and drafted the manuscript.

WQ Zhang CH Zhu, X Chen and YK Zhao conducted parts of the experiments and collected samples.

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Review

A Methodological Review on the Pharmacokinetic/Pharmacodynamic Integration of Antibacterial Drugs

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Abstract: Inappropriate application of antimicrobial agents can result in resistance by bacteria to drugs and changes in bacterial ecology. In particular, the emergence of multi-drug resistant bacteria seriously affects the antibacterial efficacy of drugs, which threatens the health and lives of humans and animals. Pharmacokinetic/Pharmacodynamic (PK/PD) models can be used to analyze the relationship between PK and PD data and the antibacterial effect. PK/PD models provide valuable guidance for optimization of dosage regimens, development of new drugs, setting of susceptibility breakpoints, and analyses of resistant mutants. The main models of PK/PD integration are *in vitro* PK/PD, *ex vivo* PK/PD, and *in vivo* PK/PD. Each of these models has its own advantages and disadvantages. Hence, knowing how to choose the appropriate PK/PD model has a huge influence on obtaining accurate PK/PD data. In this review, we describe the commonly used PK/PD methods. In this way, we provide a reference for optimizing drug regimens and preventing and controlling drug-resistant bacterial infections.

Keywords: *Antibacterial drugs, PK/PD integration model, Multi-drug resistance, Dosage regimen optimization, In vivo PK/PD model*

Antibakteriyel İlaçların Farmakokinetik/Farmakodinamik Entegrasyonu Üzerine Metodolojik Bir İnceleme

Öz: Antimikrobiyal ajanların uygun olmayan şekillerde kullanımı, bakterilerin ilaçlara direncine ve bakteri ekolojisinde değişikliklere neden olabilir. Özellikle çoklu ilaca dirençli bakterilerin ortaya çıkması, ilaçların antibakteriyel etkinliğini ciddi şekilde etkilemekte ve bu durum insan ve hayvanların sağlığını ve yaşamını tehdit etmektedir. Farmakokinetik/Farmakodinamik (PK/PD) modeller, PK ve PD verileri ile antibakteriyel etki arasındaki ilişkiyi analiz etmek için kullanılabilmektedir. PK/PD modelleri, dozaj rejimlerinin optimizasyonu, yeni ilaçların geliştirilmesi, duyarlılık sınır değerlerinin belirlenmesi ve dirençli mutantların analizleri için değerli rehberlik sağlarlar. PK/PD entegrasyonunun temel modelleri *in vitro* PK/PD, *ex vivo* PK/PD ve *in vivo* PK/PD'dir. Bu modellerin her birinin kendine göre avantajları ve dezavantajları vardır. Bu nedenle, uygun PK/PD modelinin nasıl seçileceğinin bilinmesi, doğru PK/PD verilerinin elde edilmesinde büyük bir etkiye sahiptir. Bu derlemede, yaygın olarak kullanılan PK/PD yöntemlerini açıklamaktayız. Böylelikle, ilaç rejimlerini optimize etmek ve ilaca dirençli bakteriyel enfeksiyonları önlemek ve kontrol etmek için bir referans sağlamaktayız.

Anahtar sözcükler: *Antibakteriyel ilaçlar, PK/PD entegrasyon modeli, Çoklu ilaç direnci, Dozaj rejimi optimizasyonu, In vivo PK/PD modeli*

Introduction

With the large-scale and intensive development of the animal-breeding industry, there is a risk of disease outbreaks. Hence, antimicrobial drugs are used to prevent and treat animal infections. However, inappropriate application of antimicrobial agents (e.g., inappropriate treatment course or selection of antimicrobial) can result in resistance by bacteria to drugs and changes in bacterial ecology. In particular, the emergence of multi-drug resistant bacteria

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seriously affects the antibacterial efficacy of drugs, which threatens the health and lives of humans and animals. Therefore, solving these problems is extremely important [1-3].

The most common approaches to resolve drug resistance in bacteria is to develop new veterinary drugs and optimize drug regimens. However, the speed of research and development of new drugs cannot keep pace with the mutation rate of drug-resistant bacteria. Therefore, optimization of drug regimens is a practical and reliable method to deal with the threat of drug-resistant bacteria.

An integrated pharmacokinetic/pharmacodynamic (PK/PD) model can be used to evaluate the interaction between drugs, hosts, and pathogens. This can be achieved by analyzing the relationship between PK, PD, and PK/PD parameters and the antibacterial effect, as well as predicting the values needed to elicit different antibacterial effects. Hence, PK/PD is an important method for optimizing dosage regimens [4-6].

At present, PK/PD integration model were classified to *in vitro* PK/PD, *ex vivo* PK/PD, and *in vivo* PK/PD, such as *in vitro* peristaltic model, *ex vivo* time-kill model, and *in vivo* tissue cage (TC) model. Each of these models has advantages and disadvantages. Hence, knowing how to choose an appropriate PK/PD model has a major influence on the obtained PK/PD data.

In this review, we introduced the commonly applied PK/PD methods and we this review can provide a reference for optimizing drug regimens and preventing and controlling drug-resistant bacterial infection.

Basic Concepts of PK

PK is the study of the absorption, distribution, metabolism, and elimination of drugs in the host. The main PK parameters are maximum concentration of a drug in plasma (C_{max}), time to reach C_{max} of a drug in plasma (T_{max}), time the drug concentration needs to decrease by 50% (elimination half-life ($T_{1/2\beta}$), area under the concentration-time curve (AUC), the volume of drug in the body cleared per unit time (clearance), the proportion of a drug which enters the circulation when introduced into the body and is able to have an active effect (bioavailability).

Basic Concepts of PD

PD is the study of the biochemical and physiologic effects of drugs. PD parameters describe the action of the drug upon the body and pathogens.

In terms of antibiotic therapy, the important parameters are the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), mutant prevention concentration (MPC), post-antibacterial effect (PAE), growth rate, and kill rate.

The MIC is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a micro-organism after overnight incubation. The MBC is the lowest concentration of a antimicrobial drug that will prevent the growth of an organism. The MPC can be defined as the MIC of the least susceptible single-step mutant. The PAE refers to a period of time after complete removal of an antibiotic during in which there is no growth of the target organism. The growth rate is the speed at which the number of organisms in a population increases. The kill rate is defined as the reduction of organisms numbers after interacted with antibacterials.

Basic Concepts of PK/PD Models

A PK/PD model can guide dosage regimens using PK/PD parameters which connect PK data to PD data.

The commonly applied PK/PD indices are AUC/MIC, C_{max}/MIC, %T > MIC (the percentage of drug concentration above the MIC during dosing intervals), AUC/MPC, C_{max}/MPC, and %T > MPC (the percentage of drug concentration above the MPC during dosing intervals) [7,8].

Usually, the antibacterial effect (E) is expressed by the clinical effect before and after treatment, or the change of the number of bacteria (colony forming units, CFU). In general, according to the change of bacteria population, the antibacterial effect is divided into a bacteriostatic effect (E = 0), bactericidal effect (E = -3), and eradication effect (E ≥ -4). PK/PD integration aims to establish the concentration-time-effect relationship by analyzing the relationship between PK/PD parameters and E by linear or sigmoidal formulae, and then calculating PK/PD indices for achieving different antibacterial effects.

According to the best-fitting PK/PD parameters to E, drugs are often classified into three categories [9]: (i) concentration-dependent drugs; (ii) time-dependent drugs with long PAE; (iii) time-dependent drugs with short PAE *(Table 1)*.

The antibacterial activity of concentration-dependent drugs is closely related to the drug concentration exposed to bacteria. The drug regimen requires a high dose to make the drug concentration in tissue or plasma 10-12-times higher than the MIC [10].

The antibacterial activity of time-dependent drugs is dependent upon the percentage of time the drug concentration is above the MIC during the dosing interval. Therefore, shortening the dosing interval and increasing the administration duration can ensure the required percentage of drug concentration above the MIC reaches a bactericidal effect (%T> MIC ≥ 50%) [11]. PK/PD parameters can also reflect the influence of a drug

Table 1. Classification of antibacterial drugs based on PK/PD indices			
Classification	**Antibacterial Drugs**	**PK/PD Indices**	**Activity**
Concentration-dependent	Aminoglycosides, fluoroquinolones amphotericin B, metronidazole, colistin, rifamycins	C_{max}/MIC or AUC/MIC	Primarily bactericidal
Time-dependent with short PAE	Macrolides with short half-life (erythromycin), penicillins, carbapenems, cephalosporins	%T > MIC	Primarily bactericidal
Time-dependent with prolonged PAE	Macrolides with long half-life (azithromycin, tulathromycin), clindamycin, ketolides	AUC/MIC or %T > MIC	Primarily bacteriostatic
Co-dependent	Tetracycline Glycopeptides	AUC/MIC or %T > MIC	Bacteriostatic Bactericidal

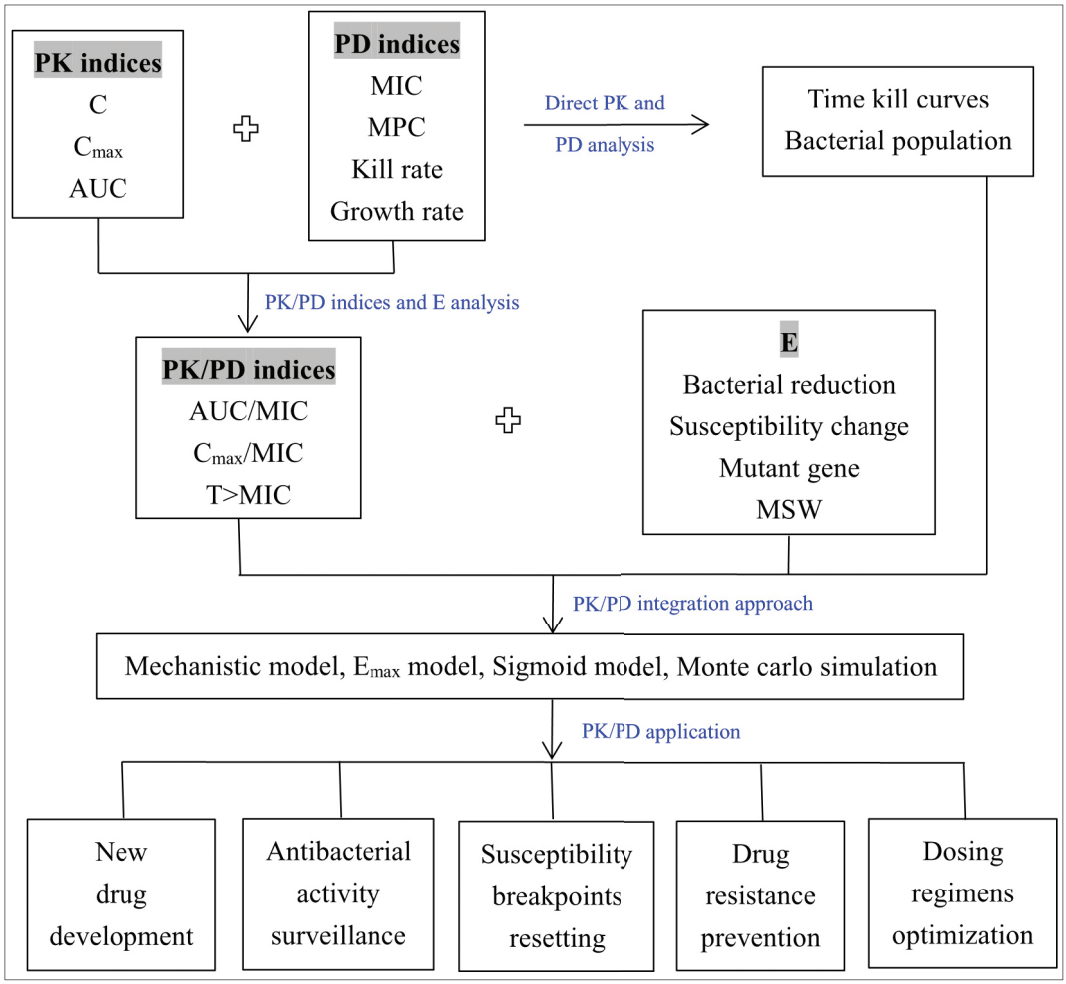

Fig 1. Process and application of PK/PD integration

regimen on the resistance of mutant bacteria. For example, the analysis of relationships between mutation frequency and PK/PD parameters based on the MPC can keep the PK/PD indices away from the resistant mutation concentration when designing the drug regimen [12]. After Monte Carlo simulation, the obtained PK/PD breakpoint (CO_{PD}) is combined to the epidemiological breakpoint (E_{coff}) or wild-type breakpoint (COWT), and clinical tipping point (CO_{CL}) to reset the susceptibility breakpoint for monitoring of bacterial sensitivity [13,14]. PK/PD integration had been used widely in several fields, and the general process and application is shown in *Fig. 1*. The commonly applied methods for PK/PD study are listed in *Table 2*.

In Vitro PK/PD Models

Static Concentration Time-Kill Curves (SCTKCs)

A SCTKC is the basic method to study the antibacterial activity of drugs and the cornerstone for developing new antibacterial agents and optimizing dose regimens. A SCTKC can be acquired by counting the bacterial population in different drug concentrations (based on

Table 2. *Classification, methods, and application of PK/PD models*

Basic Classification	Common Methods	Application
In vitro PK/PD model	Static concentration time-kill curves	Tube model
	Dynamic concentration time-kill curves	Peristaltic-pump model, Hollow-fiber model
Ex vivo PK/PD model	Tissue-cage model	Pig, calf, rabbit, sheep, goat, camel
	Serum	Most animals
	Other body fluids	Uterine fluid, ileum content, pulmonary epithelial lining fluid
In vivo PK/PD model	Animal-organ infection model	Murine, duck, chicken
	Tissue-cage infection model	Pig, calf, rabbit
	Microdialysis-based PK/PD model	Mice

different folds of the MIC) at different time points [15]. This method can directly reveal the kill rate, antibacterial effect, and recovery of bacteria. A traditional SCTKC related only to the MIC cannot reflect the detail of antibacterial activities. Therefore, two optimized SCTKC methods are applied commonly to study PK/PD integration.

One model is the kill rate-based SCTKC [16-18]. The kill rate is a PD parameter (containing the MIC and SCTKC) which represents the difference between the growth rate and death rate. The value of the kill rate is the slope of a SCTKC during different periods. This model directly reflects the antibacterial characteristic of drugs, and also can divide drugs into concentration-dependent and time-dependent types. Ferro et al.[16] studied the relationship between antibacterial concentrations and the kill rate of several antibiotics against two types of rapidly growing mycobacteria by sigmoid E_{max} fitting. The highest kill rates appeared at 24-27 h for *Mycobacterium abscessus*, and the highest E_{max} was 0.0427/h, 0.0231/h, and 0.0142/h for amikacin, clarithromycin, and cefoxitin, respectively. For *Mycobacterium fortuitum*, the highest kill rate appeared at 3-24 h and the highest E_{max} of amikacin was 0.1933/h. Cheah et al.[17] compared the difference between a kill rate-based SCTKC model with a PD metrics-free SCTKC model by analyzing the antibacterial activity of different drugs against six strains of *Acinetobacter baumannii*. They showed that both models had identical dose-response relationships. However, the kill rate-based SCTKC approach exhibited 10-times faster killing by colistin than doripenem, and the bacterial regrowth for colistin was 3-h earlier than that for doripenem. This kill rate-based model could provide a detailed framework to distinguish the antibacterial characteristics of drugs and to optimize dosage regimens. Zhang et al.[18] studied the relationship between the kill rate and doxycycline concentration against *Mycoplasma gallisepticum* at different times. Doxycycline exhibited time-dependent antibacterial activity, and the best-fitting time period was 0-48 h ($R^2 = 0.986$) and the highest kill rate was 0.11/h after PK/PD analyses.

The other model for SCTKC is to add several PD parameters with the MIC for more detailed PK/PD integration [19-21]. Nolting et al.[19] applied a modified E_{max}-model for PK/PD fitting by describing the changes in the *Escherichia coli* population over time under different piperacillin concentrations. In this model, several PD parameters were considered: maximum kill effect (K_{max}), normal growth rate (K), initial bacterial count (N), and delayed bacterial growth constant. Regoes et al.[20] established a PK/PD model comprising four PD parameters: maximum growth rate in a drug-free medium (ψ_{max}); minimum growth rate in a contained drug medium (ψ_{min}); the slope of drug concentration and kill rate (k); MICs for different bacteria (zMIC). After analyzing these PD parameters to drug concentrations (a), they showed that the higher the value of k, the greater was the antibacterial effect as identified by the MIC. Compared with use of MIC alone, this modified SCTKC-based PK/PD model containing multiple PD parameters could guide the design of the dosage regimen in more detail and more precisely.

Dynamic Concentration Time-Kill Curves

Dynamic concentration time-kill curves can simultaneously acquire dynamic antibacterial concentrations and bacterial populations in the same central compartment for PK/PD fitting by simulating the *in vivo* antibacterial PK characteristics [22]. Therefore, this model needs sophisticated equipment. The commonly used models are peristaltic model and hollow-fiber model.

A basic peristaltic model comprises one reserve compartment (containing blank medium), one central compartment (containing the drug, bacteria, and medium), and one elimination compartment (to collect the waste medium) [23]. A peristaltic pump is applied to connect each compartment and make the medium flow in turn to simulate *in vivo* antibacterial PK based on real PK data (e.g., elimination rate constant and absorption rate constant). Vadday et al.[24] applied this model to analyze the PK/PD integration of two candidate spectinamide drugs (1445 and 1599) against *Mycobacterium bovis* BCG. They showed that 1445 exhibited time-dependent antibacterial activity and the best-fit PK/PD parameter was T > MIC ($R^2 = 0.910$). For 1599, concentration-dependent antibacterial activity was

observed, and the best-fit PK/PD parameter was fC_{max}/MIC (R^2 = 0.827). However, this basic model could not prevent the loss of bacteria from the central compartment, which may affect the antibacterial effect for slow-growing bacteria and collection of mutant strains. Hence, a modified model was applied. Meletiadis et al.[25] developed a new peristaltic model for PK/PD integration. They added a dialysis tube made of semipermeable cellulose membrane which could allow free diffusion of the drug, but not bacteria. Zhang et al.[18] applied this modified model to study the PK/PD of doxycycline against *Mycoplasma gallisepticum*, and the value of %T >MIC for 0-$\log_{10}$ reduction, 2-$\log_{10}$ reduction, and 3-$\log_{10}$ reduction was 32.48%, 45.68%, and 54.36%, respectively. Nevertheless, this model also had limitations. For instance, because the pore of the semipermeable cellulose membrane was small, rapid growth of bacteria could block it, which could reduce the interaction between the drug and medium. This phenomenon could result in bacterial death because of a lack of nutrients after long-term incubation. Therefore, suitable pathogens and culture duration are needed for application of this model.

Hollow-fiber models are of small size and sophisticated build quality. The central chamber contains thousands of hollow fiber tubes which can prevent bacterial loss and are often used to simulate antibacterial PK characteristics according to a multi-compartment model [26]. Jacobsson et al.[27] established a hollow-fiber infection model to study the PK/PD of zoliflodacin against susceptible and resistant *Neisseria gonorrhoeae*. A dose <1 g/day could result in treatment failure and lead to drug-resistant mutations *(gyrB)* in bacteria. A dose >2 g/day could eradicate this effect for both strains. Bhagunde et al.[28] applied a hollow-fiber infection model to study the PK/PD of relebactam against imipenem-resistant *Pseudomonas aeruginosa*. *f*AUC/MIC was the best-fitting PK/PD parameter for an antibacterial effect, and *f*AUC/MIC = 7.5 mg·h/L could produce a 2-$\log_{10}$ reduction.

The advantages of these *in vitro* time-kill-related PK/PD models are that they are simple, economical, easy to operate, and reflect antibacterial activity directly. Upon optimization of PD parameters, these models can guide the development of new drugs and optimization of dosage regimens. The disadvantage of these *in vitro* time-kill-related PK/PD models is that the antibacterial effect does not reflect the influence of the host on drugs and pathogens.

Ex Vivo PK/PD Models

Ex vivo PK/PD integration has been employed to study the antibacterial effect (time-kill curves and MIC) of drugs against pathogens in biological matrices (serum, tissue fluid, inflammatory exudates). After drug administration, the matrix is collected at different time points and divided equally into two samples. One sample is applied to detect the drug concentration for PK study. The other sample is added logarithmic pathogenic bacteria for time kill curves study. The PK/PD parameter used is AUC_{24h}/MIC (the antibacterial concentration in the matrix multiplied by 24 h and divided by the MIC).

A serum sample can be collected readily, so has been applied widely for *ex vivo* PK/PD [29,30]. Li et al.[29] studied the *ex vivo* PK/PD of ceftiofur against *Haemophilus parasuis* in porcine serum. They documented that the values of AUC_{24h}/MIC to produce bacteriostatic, bactericidal, and clearance effects were 36.006 h, 71.637 h, and 90.619 h, respectively. Lee et al.[30] studied the *ex vivo* PK/PD of levofloxacin against *Escherichia coli* in broiler chickens. Levofloxacin showed concentration-dependent antibacterial activity against a clinical isolate of *E. coli* (MIC = 0.125 μg/mL) and the predicted dosage to produce a bactericidal response and bacterial eradication was 2.9 mg/kg/day and 4.3 mg/kg/day, respectively. However, the serum samples reflect the changes in drug concentration and cytokines in the systemic circulation, which are different to the changes in target tissues. Hence, it is more rational to use samples collected from target tissues (e.g., tissue fluid).

To acquire tissue fluid, a TC model in animals is used commonly. The TC can be cylindrical or spherical. Cylindrical TCs are smaller, can collect less tissue fluid, and are often used in tight-skin animals (e.g., pigs) [31]. Spherical TCs are larger in volume, can obtain more tissue fluid, and are often used for loose-skin animals (e.g., goats, camels) [32,33]. This model is established by surgery to implant the sterile TC between skin and muscle. About 4-weeks later, granulation tissue surrounds the TC and secretes tissue fluid into the cage. Zhang et al.[34] applied a porcine TC model and studied the PK/PD of cefquinome against *E. coli* (MIC_{90} ≤ 0.50 μg/mL). The AUC_{24h}/MIC to produce bactericidal and eradication effects was 35.01 h and 44.28 h, respectively. The TC model has been applied to study many antibacterial drugs and pathogens. However, this model has some disadvantages. For instance, this model is not suitable for the study of poorly absorbed drugs because such drugs have difficulty reaching the systemic circulation. Also, intracellular pathogens cannot be applied to this model because the TC sample is extracellular fluid.

In addition to the *ex vivo* PK/PD models described above, other matrix samples have been studied. Maan et al.[35] investigated the PK/PD of aditoprim against *Trueperella pyogenes* in the uterine fluid of cattle. They documented an AUC_{24h}/MIC to produce bacteriostatic, bactericidal, and clearance effects to be 2904 h, 13047 h, and 21970 h, respectively. Lei et al.[36] applied the content of porcine ileum to study *ex vivo* PK/PD of marbofloxacin against *E. coli*. They revealed the AUC_{24h}/MIC to produce bacteriostatic, bactericidal, and clearance effects to be 16.26 h,

23.54 h, and 27.18 h, respectively. Luo et al.[37] applied the fluid from pulmonary epithelial linings to study the *ex vivo* PK/PD of ceftiofur against *Streptococcus suis*, and provided abundant reference data for the design of dosage regimens.

Compared with the *in vitro* model, the *ex vivo* PK/PD model can reflect (at least in part) the effects between drugs, the host (e.g., immune factors, defense factors) and pathogenic bacteria. Such information provides detailed understanding of the influence of body substances on pathogenic bacteria. In addition, the *ex vivo* PK/PD model requires fewer/less damage to animals and lower costs, and is an important supplement to the *in vitro* PK/PD model. However, the *ex vivo* PK/PD model has limitations. For example, although this model applied *in vivo* PK data, but the PD was still acquired from static drug concentrations which not synchronized with the absorption and elimination of drugs in the host.

In Vivo PK/PD Models

This model applies *in vivo* PK and PD data for PK/PD integration. It can be used to analyze the interaction between drugs, pathogens, and the host. The most commonly used model types are the animal-organ infection model (AOIM) and tissue-cage infection model (TCIM).

AOIM

The AOIM is the most commonly applied *in vivo* PK/PD model. The basic research approach involves division into four stages. First, an infection model in a target organ is established to fit the linear relationship between the dose and drug concentration in plasma by administrating a different antibacterial dose on one occasion. Second, the antibacterial effect is calculated after therapy for 24 h at different intervals and dosages by dose fractionation. Third, an extrapolation approach is applied to acquire the drug concentrations of each dose during 24 h for calculation of PK/PD parameters. Finally, a sigmoid inhibitory E_{max} formula is used to analyze the relationships between PK/PD parameters and antibacterial effect, and to guide regimen design.

The AOIM has been established in mouse thighs, mouse lungs, chicken lungs, and duck lungs [38-40]. Xiao et al.[38] applied a duck-lung infection model to study the PK/PD of florfenicol against *P. multocida*. The AUC_{24h}/MIC to produce a bactericidal effect was 108.19 h and 54.30 h against the strains 0825Y1 and 0901J1, respectively, and the recommended dose was 52 mg/kg after Monte Carlo simulation. Zhang et al.[39] established a chicken-lung infection model to study the PK/PD of danofloxacin against *Mycoplasma gallisepticum* and analyzed the relationships between PK/PD parameters and resistant mutant genes. Li et al.[41] applied a mouse-lung infection model to study the PK/PD of nemonoxacin against *Streptococcus pneumoniae*. The AUC_{24h}/MIC was 8.6 h, 23.2 h, and 44.4 h to reach a bacteriostatic effect, 1-log_{10} reduction, and 2-log_{10} reduction, respectively. Watanabe et al.[42] used a mouse-thigh infection model to study the PK/PD of teicoplanin against *Staphylococcus aureus*. The $fAUC_{24h}$/MIC to produce a bacteriostatic effect and 1-log_{10} reduction was 54.8 h and 76.4 h, respectively.

The antibacterial effect of the AOIM is the result of the interaction between the host, bacteria, and drugs in the target organ, which is similar to clinical treatment. However, the AOIM has three main shortcomings. First, the PK data are obtained from plasma and are meant to represent the target organ. Some researchers have studied the relationships between plasma and target-organ values, but the target-organ data were not applied extensively. The other deficiency is that the PK data was consist with plenty of independent animals which may be influenced from the difference of animals physical condition. Third, the antibacterial effect is defined as the change in the final bacterial population 0 h and 24 h after treatment, which does not reflect the dynamic change in bacterial populations.

TCIM

After the TC model has been established, a certain concentration of bacteria solution (10^8-10^{10} CFU/mL) is injected and incubation permitted for a period of time. When the bacterial population stabilizes at 10^6-10^8 CFU/mL, then the TCIM has been established. After a series of dose regimens have been administrated, the TC fluid is removed at different time points for measurement of drug concentrations and bacterial populations. Finally, the relationships between PK/PD parameters and the antibacterial effect are analyzed to guide the design of the dosage regimen. This model realizes PK/PD integration at the same location and reflects the interactions between the host, drugs and bacteria. Hence, this infection model has been used widely for PK/PD integration and been established in pigs, rabbits, and cows [43-45]. Cao et al.[44] applied a calf TCIM to study the PK/PD of marbofloxacin against *P. multocida*. The AUC_{24h}/MIC to produce a 1.5-log_{10} reduction and 3-log_{10} reduction was 18.6 h and 50.65 h, respectively. Xiong et al.[46] applied a rabbit TCIM to study the relationship between the PK/PD parameters of cefquinome against mutant strains of *S. aureus*. They revealed that resistant *S. aureus* were selected and enriched if %T > MPC < 58% or %T > MIC_{99} ≥ 70%.

The TCIM has two main limitations. First, this model is based on a local infection between skin and muscle, which is not the target organ for all pathogens. Therefore, the obtained results are different from those after a systemic infection. Second, TC fluid is a component of extracellular fluid, which is suitable for most bacterial growth. Hence,

this model is not applicable for the study for cell-growing microorganisms (e.g., *Mycoplasma* species).

Microdialysis-Based PK/PD Model

Microdialysis is a microsampling technique based on dialysis. It permits sampling of low quantities, is associated with little damage to tissue, and enables continuous sampling and real-time monitoring of drug concentrations. The basic constituents of a microdialysis system are a micro-control pump, microsyringe, inlet line, microdialysis probe, outlet line, and collection tube. The microdialysis probe comprises a semipermeable membrane. The levels of substances either side of the semipermeable membrane can be balanced by passive transport. The micro-control pump is used to make the perfusate flow slowly through the dialysis membrane and bring-out the dialysate. After detected the drug concentration in the dialysis, the concentration in organ was converted by *in vivo* recovery. This method is very important for acquisition of the PK parameters of target organs (site of bacterial infection), so it has been applied for PK studies. Bernardi et al.[47] used microdialysis to investigate the penetration of tobramycin from the plasma to the lungs in rats. Yang et al.[48] employed microdialysis to study the PK of florfenicol in pig lungs. Zhang et al.[49] applied microdialysis to study the influence of body status on cefquinome PK in mouse thighs. Hence, microdialysis technology has important applications in PK/PD research, especially for the AOIM, because PK parameters in target organs can be obtained continuously to allow more precise PK/PD integration. Studies on the PK/PD integration of antibacterial agents using microdialysis have been reported rarely. Zhang et al.[50] established a mouse-thigh microdialysis infection model to study the PK/PD of cefquinome against *Actinobacillus pleuropneumoniae*. The %*f*T >MIC to produce a 1-$\log_{10}$ reduction, 2-$\log_{10}$ reduction, and 3-$\log_{10}$ reduction was 36.11%, 52.96%, and 82.68%, respectively. Microdialysis could have broad application prospects in PK/PD integration.

Conclusions and Recommendation

There is an increasing prevalence of drug resistance and slowing down of new-drug development because of the expense of research and development. Hence, PK/PD models can be used to guide optimization of dosage regimens which can prolong the life of antibacterial drugs, increase the antibacterial effect, and prevent the emergence and spread of resistant bacteria. Various PK/PD models have their own advantages and disadvantages, so the selection of an appropriate PK/PD model has a huge influence on obtaining accurate results. With the development and application of new technologies, more accurate PK/PD results will be obtained to guide appropriate use of clinical drugs.

Availability of Data and Materials

Not applicable.

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Competing Interest

The authors declare that they have no conflict of interest.

Author's Contributions

HW and LZ contributed to the methodology, software use, validation, and writing. LZ and JH contributed to the supervision.

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